Retrograde signalling within fear neurocircuitry: Nitric oxide signalling from the lateral nucleus of the amygdala regulates thalamic EGR-1 mediated alterations of presynaptic protein levels during auditory fear conditioning

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A thesis submitted in partial fulfilment of the requirement for the Degree of Doctor of Philosophy at the University of Canterbury by Kathie A. Overeem

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University of Canterbury

2009

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Acknowledgements

Firstly, I would like to extend my deepest thankfulness to Professor Glenn Schafe for his guidance and support. I would also like to thank Professor Rob Hughes for his co-supervision. To the students and employees of the Schafe lab- thanks for your assistance and much appreciated support. Over the course of my doctoral studies I was extremely fortunate to be awarded a number of awards and scholarships that made my stay in the United States and research at Yale University possible. I extended my gratitude to the following New Zealand based organizations that made it all possible: Fulbright, Ministry of Research and Science, Education New Zealand and the Tertiary Education Commission.

Abbreviations

ACSF	Artificial cerebral spinal fluid
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANP	Atrial natriuretic peptide
ARC	Activity regulated cytoskeleton
BDNF	Brain derived neurotrophic factor
BSA	Albium from bovine serum
CE	Central nucleus of the amygdala
CaM kinase	Calcium-calmodulin dependent kinase
cAMP	Cyclic adenosine monophosphate
CNP	C-type natriuretic peptide
cPTIO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
CRE	Cyclic AMP response element
CREB	cAMP response element binding protein
CS	Conditioned stimulus
DA	Dopamine
DAB	3,3'-diaminobenzidine
DMSO	Dimethyl sulfoxide
EGR-1	Early-growth-response gene 1
ELK1	Ets-like gene 1
ERK	Extracellular regulated kinases'
FG	Fluorogold
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Guanylate (or guanylyl) cyclase
IEG	Immediate early gene
IHC	Immunohistochemistry
ISI	Interstimulus interval
IP	Intraperitoneal
LA	Lateral nucleus of the amygdala
LTM	Long-term memory
LTP	Long-term potentiation
MAPK	Mitogen activated protein kinase
MDMA	3,4-methylenedioxymethamphetamine
MGM	Medial nucleus of the medial geniculate
MGV	ventral region of the medial geniculate

Abbreviations continued

mRNA	Messenger ribonucleic acid
NE	Norepinephrine
NMDA	*N*-methyl *D*-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
ODN	Oligonucleotide
PB	Phosphate buffer
PBS	Phosphate buffered saline
PI-3	Phosphatidylinositol 3
PIN	Posterior intralaminar nucleus
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PPF	Paired pulse facilitation
PP	Peripeduncular nucleus
PSD-95	Post synaptic density 95
SRE	Serum response element
STM	Short-term memory
US	Unconditioned stimulus
VAMP	Vesicle associated membrane protein family
7ni	7-nitroindazole

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Abstract

Previous research has shown that nitric oxide signalling in the lateral nucleus of the amygdala is required for the consolidation of Pavlovian conditioned fear. Given the evidence that nitric oxide can act as a retrograde signalling molecule in *in vitro* models of memory consolidation the question arises whether this is also occurring within behavioural memory models? Using auditory fear conditioning this research shows that nitric oxide does indeed act as retrograde signalling molecule in the fear system. Its synthesis in the lateral nucleus of the amygdala regulates conditioning induced expression of the immediate early gene early growth response gene 1 (EGR-1) in cells of the auditory thalamus that project to the lateral nucleus of the amygdala. The regulation of EGR-1 expression by the lateral nucleus of the amygdala was proven to be dependent on amygdala-based cellular excitation, nitric oxide synthesis and NR2B-NMDA receptor activation but not ERK/MAPK activity. Using an EGR-1 antisense oligonucleotide to prevent training induced EGR-1 expressions in the auditory thalamus it was shown that this gene upregulation is necessary for the consolidation of conditioned fear. Finally, inhibition of EGR-1 upregulation in the auditory thalamus was proven to impair conditioning induced increases in the presynaptic proteins synaptophysin, and synapsin II and II back in the lateral nucleus of the amygdala. Overall, the results of this dissertation have shown that nitric oxide acts as a retrograde messenger in a mammalian memory system by modulating gene expression in presynaptic cells. This modulation of gene expression serves to increase levels of presynaptic proteins back at the origin of nitric oxide synthesis. This supports the long standing doctrine that nitric oxide acts as a retrograde signalling molecule to coordinate presynaptic changes associated with memory formation.

Chapter 1

Introduction

Structure of this thesis

The following chapter is a brief introductory chapter. It begins by describing Pavlovian fear conditioning, followed by a description of short-term memory (STM) and long-term memory (LTM). Next the intracellular signalling mechanisms pertinent for each form of memory are examined. It must be noted, that an emphasis is put on introducing signalling mechanisms, or intracellular machinery, that are specifically relevant to the research conducted in this thesis. The following chapters (2-6) present individual sets of experiments that were undertaken for this dissertation. Each chapter examines the role, or regulation, of the immediate early gene (IEG) early-growth-response gene 1 (EGR-1) in the auditory thalamus for auditory fear conditioning. Each includes an in-depth introduction and discussion, which elaborates on information presented within the following introduction. The final chapter concerns the discussion of this thesis, where the results of this research and research limitations are considered in light of previous research pertaining to mammalian memory formation.

General introduction

Pavlovian fear conditioning is a popular paradigm used by neuroscientists who endeavour to understand the neuroanatomical mechanisms that underpin fear memory formation. During the conditioning procedure an emotionally-neutral conditioned stimulus (CS) obtains the value of an innately aversive unconditioned stimulus (US). Because of the adaptive nature of fear memory formation, the association is quickly and robustly learned by an animal in as little as one CS-US pairing. Neuroanatomically, researchers using ablation, pharmacological, and electrophysiological research methods

have revealed that the key locus of fear memory representation resides in the lateral nucleus of the amygdala (LA) (For examples see Huff, 2004; Schafe, 2005). In essence, convergence of neurotransmission associated with both the CS and US within the LA is thought to potentiate synapses conveying CS-associated neurotransmission. As a result, subsequent presentation of the CS alone results in a fear response via activation of the central nucleus of the amygdala (CA). Thus, conditioning forms a memory association that imbues the CS with fearful affect. The molecular mechanism that underpin the formation of this association parallel those that underpin the formation of various other forms of memories not only in mammalian nervous systems but also in invertebrate systems (Bailey, 1996). Thus, fear memory formation is an excellent tool for studying memory formation in general. Because of the similarities between memory paradigms the following introduction, while predominantly discussing research concerning fear conditioning, will often mention research findings from a variety of memory paradigms, including research concerning *in vitro* examinations of synaptic plasticity, where long-term-potentiation (LTP) of synapses in either the hippocampal or amygdala tissue is used as a model of memory formation.

The dynamics of memory formation

Memory formation, working memory aside, is often divided into two stages (however, see Stough, 2006). Firstly, there is STM that lasts for minutes to hours. It is subject to stabilization and because of such is susceptible to interference or loss. Secondly, there is LTM, or consolidated, memory, which last for hours or more and is characteristically resilient to interference. At a molecular level, STM is associated with covalent modifications of pre-existing proteins at synapses that constitute the memory trace. On the other hand, LTM is associated with a reprogramming of gene expression

and *de novo* protein synthesis, which is thought to lead to long-term structural alterations at synapses. Both STM and LTM are associated with an increase in synaptic connectivity or efficacy at connections between neurons, albeit via different mechanisms (Moser, 1999). This notion was firstly, and most famously, suggested by Hebb in the 1940's, when he proposed that synaptic connections between two cells would be strengthened if both were active simultaneously. Behaviourally, the only means by which a researcher can be sure that memory formation has occurred is if recall at a later time is successful. Enhanced synaptic efficacy is thought to support memory retrieval by endowing subsequent neurotransmission with a privileged pathway within the cells that constitute the memory engram.

Molecular mechanisms of memory formation

After sufficient cellular excitation, an increase in intracellular calcium within LA neurons' sets in motion the molecular events required for fear memory formation. There are a number of means by which this can occur such as liberation from intracellular stores and influx from the extracellular space. Activation of ligand-gated ionotropic receptors allows for a rapid and localized increase in calcium. One such receptor renowned for its role in memory formation is the glutamatergic *N*-methyl *D*-aspartate (NMDA) receptor. This receptor is referred to as a coincidence detector because it opens only when both the pre- and post-synaptic cell have been activated simultaneously (Bauer, 2002; Campeau, 1992; Fanselow, 1994; Lee, 1998; Miserendino, 1990). A more detailed description of the dynamics of the receptor will be presented in Chapter 4. At this point it is sufficient to say that there is a plethora of research showing that an increase in intracellular calcium concentration, at least in part, through NMDA receptors is critical for the acquisition and subsequent consolidation of

a LA-based fear memory representation. Intracellular calcium acts as a second messenger, amplifying the extracellular signal by activating subsequent second messengers, which also, in turn, diffuse through the intracellular space activating their target proteins.

One of the prime targets of second messengers are protein kinases. These phosphotransferases catalyze the transfer of phosphate groups to target proteins consequently changing their structural conformation. At this time, the protein is termed "phosphorylated" or "activated". During memory formation the first role these kinases serve is to temporarily increase synaptic efficacy leading to STM. More specifically, they change the activity of the synapse by phosphorylating synaptic proteins, such as glutamatergic alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, consequently leading to their increased conductance properties and therefore increase excitatory activity at the synapse. Further, the actions of kinases contribute to exocytosis of glycoprotiens that participate in cell adhesion, altering connectivity between the postsynaptic and presynaptic cell (Izquierdo, 2002). Such processes appear to hold a memory in an archival type state until cellular activity associated with LTM can take place. Hippocampal LTP research has revealed that during the early phases of synaptic potentiation a protein-synthesis-independent synaptic tag that last around an hour is activated. This tag is thought to sequester LTM associated *de novo* synthesized proteins to the tagged synapses (Frey, 1997, 1998). It appears that activation of both the NMDA receptor and/or ryanodine receptors is required for this process (Martin, 2001; Sajikumar, 2009; Shi, 1999). If the tag is lost before processes associated with LTM can take place, then the memory trace is also lost forever. During the memory formation the activity of protein kinases is enhanced suggesting that the tag may be dependent or at least related to protein kinase activity.

A large number of kinase signalling cascades that subserve memory consolidation have been identified, including protein kinase A (PKA), protein kinase C (PKC), protein kinase G (PKG), phosphatidylinositol 3 (PI-3) kinase, calcium-calmodulin dependent kinase (CaM kinase) and mitogen activated protein kinase (MAPK) (Blaeser, 2006; Di Benedetto, 2009; Lin, 2001; Ota, 2008; Rodrigues, 2004; Schafe, 2000; Sui, 2008). All kinases are either directly or indirectly activated by an increase in intracellular calcium concentration. As a result, their activation is often transient; however, some remain active long after intracellular calcium concentrations return to normal basal levels. These kinases are thought to play a key role in the transition of a memory from an ephemeral short-term state to a consolidated form. One such family of protein kinases is the MAPK signalling cascade.

The MAPK pathway is comprised of three serially linked, highly conserved across species, protein kinases. Upstream of the pathway is the membrane bound G-Protein Ras, which binds and recruits the initial signalling component of the cascade; Raf. A membrane bound Raf is capable of activating the second core component of the pathway MEK. The final components of the cascade are the extracellular regulated kinases' (ERK); 1 and 2 (Davis, 2006). ERK 2, however, appears to be the more predominantly required ERK for fear memory formation (Selcher, 2001). Nonetheless, when phosphorylated the ERK's act as nuclear shuttle proteins. They translocate to the nucleus and activate transcription factors (discussed below), essentially setting in motion gene transcription that ultimately leads to *de novo* protein synthesis (for a review see Chang, 2001). Accordingly, the MAPK cascade has been implicated in processes such as cellular proliferation and differentiation and, most importantly with regards to this thesis, memory consolidation. However, it should be noted the MAPK cascade, as with many of the other signalling pathways, is subject to extensive cross

talk. This means that activation of this kinase pathway does not occur in serial isolation. More specifically, the MAPK pathway, specifically Raf, is thought to be the point of convergence for, or at least down stream of, other kinase pathways such as PKG, PKA, PKC, PI-3 kinase and CaMK (Impey, 1998; Lin, 2001; Micheau, 1999; Ota, 2008; Roberson, 1999). Thus, while it is erroneous to state that one particular kinase signalling cascade is key for fear memory consolidation, it appears that the MAPK pathway is one of the more pivotal pathways.

One of the end results of kinase activation is a change in activity of transcription factors. A transcription factor binds to specific sequences of DNA and controls the transfer, or more specifically, transcription of genetic DNA to messenger ribonucleic acid (mRNA) (Karin, 1990; Latchman, 1997; Lee, 2000; Nikolov, 1997; Roeder, 1996). Messenger-RNA is, in turn, translated into a protein product by ribosomes. This occurs outside of the nucleus in the cytoplasm of the cell. Two well known transcription factors are 1) the constitutively expressed Cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) and, 2) Ets-like gene 1 (ELK1) (Davis, 2000). Activation of these transcription factors is, in turn, dependent on NMDA receptor activation and subsequent activation of kinase signalling pathways including MAPK (Cammerota, 2000; Davis, 2000; Deisseroth, 1998; Lamprecht, 1999).

The first phase of gene expression associated with memory formation occurs over minutes or even hours (Tischmeyer, 1999). The protein products of these genes have been coined immediate early genes or IEGs. They, in turn, can be divided into two categories; 1) direct effectors, which are thought to change the structure or function of the cell, and 2) transcription factors (Clayton, 2000). The latter are transiently increased and return to the nucleus of the cell where they induce gene transcription of late response genes (Clayton, 2000). A number of IEGs have been implicated in fear

memory consolidation, including activity regulated cytoskeleton (ARC) gene, c-FOS, and the early growth response gene 1 (EGR-1). These genes are thought to play a role in memory consolidation as they are upregulated in the LA after contextual or auditory fear conditioning (Malkani, 2000; Ploski, 2008; Scicli, 2004). Further, contextual fear conditioning can increase cFOS and ARC levels in the hippocampus (Huff, 2006; McIntyre, 2005), suggesting that IEG upregulation can occur in the wider fear circuitry, specifically in an area with dense projections to the LA.

The IEG researched in this thesis is EGR-1. It acts as a transcription factor and has been implicated in the consolidation of various forms of memory (Bozon, 2002; Davis, 2003; Davis, 2000; Jones, 2001). This IEG will be introduced in greater detail in Chapter 2. Because, in part, of the action of IEG-transcription factors, such as EGR-1, there is more than one peak of gene expression during the early phase of memory formation (Clayton, 2000; Grecksch, 1980). As mentioned, this reprogramming of gene expression within the cell is thought to contribute to permanent functional alterations at synaptic connections between neurons that constitute a memory trace.

Synaptic structural alterations associated with memory formation

Structurally, memory consolidation is thought to involve the growth or restructuring of both presynaptic terminals and dendritic spines. This includes the perforation or growth and division of the postsynaptic density, geneses of new presynaptic terminals, insertion of new receptors into the membrane, facilitated neurotransmitter release and accordingly an increase in the density, or at least a significant redistribution, of synaptic proteins required for the abovementioned processes (Calverley, 1990; Ehninger, 2005; Lamprecht, 2006; Rumpel, 2005; Yu, 2008). At a behavioural level, researchers utilize the increase in protein density as a

measure of learning induced structural alterations at synapses within the LA. For example, fear conditioning is known to increase the protein levels of spinophilin and profilin, a protein localized to spine heads and involved in synaptic growth respectively (Lamprecht, 2006). In the same manner, conditioning-induced increases in presynaptic proteins have been observed (Nithianantharajah, 2008). More specifically, Nithianantharajah and colleagues (2008) have reported a significant increase in the synaptic vesicle protein synaptophysin relative to tone-alone, shock-only and context-only controls. As mentioned above, an increase in the level of a particular intracellular protein must lie downstream of kinase and transcription factor activation. This suggests that activation of presynaptic intracellular pathways must subserve these presynaptic alterations that are associated with fear memory formation.

Presynaptic underpinnings of fear conditioning

With regard to auditory fear conditioning, cells within the nuclei of the auditory thalamus that process auditory information send projections to the LA, and thus constitute an essential presynaptic component of the LA. The auditory thalamus is typically comprised of the peripeduncular nucleus (PP), posterior intralaminar nucleus (PIN), and the medial nucleus of the medial geniculate (MGm). Researchers examining the role of this area in auditory conditioning have reported that blockade of either ERK/MAPK signalling or mRNA synthesis prevents auditory fear memory consolidation (Apergis-Schoute, 2005). Thereby, revealing that activation of presynaptic intracellular signalling cascades is, indeed, required for fear memory formation. Furthermore, there is research that suggests that this intracellular activity is occurring to support the formation of an LA-based fear memory representation. More specifically, research has shown that activation of ERK/MAPK in the auditory

thalamus is required for LTP-induced plasticity in the LA (Apergis-Schoute, 2005). However, the opposite does not occur; when ERK/MAPK activity is blocked in the LA, auditory thalamic plasticity remains unaffected (Schafe, 2005). This finding, in light of the requirement for LA-based ERK signalling in auditory fear memory consolidation (Schafe, 2000), reveals that plasticity in the auditory thalamus is not sufficient for the formation of a fear memory representation, but instead serves to support the development of a LA-based memory (Apergis-Schoute, 2005). Thus, there is a growing body of literature that illustrates a presynaptic component to fear memory consolidation, whereby synaptic plasticity is accompanied by both a presynaptic and postsynaptic change in genomic expression and subsequent synaptic protein levels. Ultimately, this activity is thought to result in functional alterations at presynaptic terminals. Indeed, in line with this, there is research that suggests that conditioning can potentiate presynaptic signalling at thalamo-LA synapses.

Researchers examining paired pulse facilitation (PPF) have reported enhanced transmitter release probability in fear conditioned animals (McKernan, 1997; Zinebi, 2003). During the PPF protocol the presynaptic cell is stimulated by two pulses of excitation identical in all aspects. If the interval between the two pulses is sufficiently brief the second of the two stimuli evokes a larger presynaptic response than the first. PPF is generally used as a tool to examine presynaptic changes in transmitter release probability. In line with the importance of NMDA receptor activation in fear memory formation, PPF at thalamo-LA synapses is dependent on postsynaptic NMDA receptor activation (Zinebi, 2001). Given, the postsynaptic location of the NMDA receptor a retrograde signalling molecule is thought to bridge the gap between this postsynaptic receptor activation and training induced changes in presynaptic activity. In the mammalian brain, a number of signalling molecules with retrograde signalling

capabilities exist including carbon monoxide and nitric oxide (NO) (Dawson, 1994). In the amygdala, NO signalling, or downstream targets of this molecule, is required for successful conditioning to both a light and tone CS (Ota, 2008; Overeem, 2006; Schafe, 2005). Thus, suggesting that NO could be acting in a retrograde type manner at thalamo-LA synapses and contributing to conditioned induced changes in presynaptic activity.

Nitric oxide

Nitric oxide is the most studied retrograde signalling molecular in the central nervous system. It is capable of diffusing across both aqueous and lipid environments in a unrestricted and consequently isotropic manner (Arancio, 1996; O'Dell, 1991). The diatomic gas is catalysed from L-arginine, by the enzyme nitric oxide synthase (NOS). An increase in intracellular calcium concentrations leads to activation of the calcium/calmodulin signalling complex, which, in turn, activates NOS. In the brain three isoforms of NOS have been indentified 1) neuronal, 2) epithelial and 3) inducible. Neuronal-NOS is indirectly attached to the NMDA receptor and is thus thought to be a mediator, or more specifically, second messenger of NMDA receptor-mediated processes. The enzyme is linked to the receptor via the scaffold protein post synaptic density 95 (PSD-95). This attachment assists in the formation of a synaptic signalling complex involving the NMDA receptor, nNOS, and calmodulin (Fukunaga, 2005). This functional interaction between closely associated proteins serves to precipitate NO synthesis as a result of calcium influx through the activated receptor (Kiss, 2001). As a signalling molecule, NO interacts directly with iron, copper, or iron-sulphur moieties within larger metalloprotiens. These targets of NO can be broadly defined as guanylate (or guanylyl) cyclase (GC) dependent or independent (Krumenacker, 2004). In the

latter, NO directly modifies proteins through nitration or s-nitrosylation, while in the former it acts through the production of the second messenger cGMP and subsequent activation of protein kinase G (PKG) (Contestabile, 2008). Recently, the requirement for NO signalling in fear memory consolidation has been attributed, at least in part, to PKG signalling (Ota, 2008). However, NO's role in fear memory consolidation has not been explicitly attributed to presynaptic modulation of intracellular signalling.

This current research

The primary aim of this study was to examine whether the amygdala drives gene expression in the auditory thalamus via NO signalling during auditory fear conditioning. Within the experiments a three tone-shock pairing paradigm was used for conditioning, animals were sacrificed 2-24 hours after training; protein changes in the auditory thalamus or LA were then examined. As a result, it was not possible at a behavioural level to examine whether the animals had specifically learnt the association. However, as mentioned above, the association can be learnt in as little as one CS-US pairing. Further, there is a plethora of research indicating that three tone-shock pairings is suffice for the formation of a robust fear memory association.

Overall, the research of this dissertation shows that the LA drives the expression EGR-1 in the auditory thalamus during auditory fear conditioning. The results show that NO acts as a retrograde signalling molecule in the fear circuitry to mediate this effect. Furthermore, this thalamus based genomic regulation is then linked back to alterations in thalamic cell terminals located within the LA. The experiments for this dissertation are divided into 5 Chapters.

1) Examination of training induced EGR-1 upregulation in the auditory thalamus (Chapter 2)

The research conducted for this thesis begun by examining conditioning-induced gene upregulation in the auditory thalamus. As discussed above, while activation of intracellular signalling cascades in this area is required for fear memory consolidation, no research has revealed a specific change in genomic activity. In this research the IEG EGR-1 was examined. It is known to be located downstream of the MAPK signalling pathway, the activity of which in auditory thalamic cells is necessary for both fear memory consolidation and synaptic plasticity in the LA. Furthermore, EGR-1 is known to play an important role in memory formation and itself acts as a transcription factor to regulate further genomic modifications. Thus, its activity could have a widespread effect on the levels of numerous presynaptic proteins.

2) Amygdala modulation of training induced thalamic EGR-1 expression (Chapter 3, sections A and B)

In the next series of experiments pharmacological manipulations were used to modify LA-based cellular processes. The affect this had on training induced EGR-1 expression was examined. The pharmacological manipulations were $GABA_A$ activation with muscimol, NO antagonism with the selective nNOS inhibitor 7-ni and the membrane impermeable NO scavenger cPTIO, inhibition of the MAPK signalling pathway using the selective ERK1/2 inhibitor U0126, and blockade of NR2B-NMDA receptor activation with ifenprodil

3) Double label Fluorogold-EGR-1 tract-tracing experiment (Chapter 4)

The anatomical connectivity between thalamic cells expressing EGR-1 and LA projection cells was determined using the retrograde tracer Fluorogold. This series of experiments examined whether training-induced EGR-1 expression observed in the auditory thalamus actually occurred within LA projection cells.

4) Examination of the requirement for EGR-1 upregulation for auditory fear conditioning (Chapter 5)

Next, the necessity for EGR-1 protein upregulation in the auditory thalamus for fear memory consolidation was examined. A selective EGR-1 antisense-oligonucleotide was microinfused into the auditory thalamus in order to prevent preventing training induced EGR-1 upregulation. Previous research has shown that EGR-1 upregulation in the LA is required for fear memory consolidation. This series of experiments examined whether its presynaptically upregulation, relative to the LA, necessitates the consolidation of an auditory fear memory association.

5) Examination of presynaptic alterations (Chapter 6).

Finally, training induced upregulation of presynaptic proteins was examined. This series of experiments sought to, in part, replicate the above mentioned finding that auditory fear conditioning increases the amount of synaptophysin protein in the LA. In addition, conditioning induced regulation of other synaptic vesicle associated proteins: synapsin I and II was also examined. Finally, intra-thalamic EGR-1 antisense-oligonucleotide microinfusions were used to show that EGR-1 upregulation in the auditory thalamus is upstream of presynaptic alteration of cellular constitutes in the LA.

Chapter 2

Training-induced upregulation of Thalamic EGR-1

Over the last few years there has been a dramatic increase in the amount of research concerning the molecular mechanisms that underpin the acquisition and consolidation of auditory conditioned fear. The primary area of interest for most has been the LA, where gains in synaptic connectivity are thought to gate the ability of the CS to induce a fear response via activation of the CE (Rogan, 1997). Although there is a great deal of research suggesting that the LA is the pivotal neuroanatomical location of a fear memory association other brain areas are known to play a role.

Plasticity within the auditory thalamus

The auditory thalamus is comprised of 1) MGM, 2) PIN and 3) the PP (see Figure 1). These structures send dense neuronal projections to the LA, and thus relay neurotransmission associated with auditory stimuli to the amygdala (Bordi, 1994; Doron, 1999; Farb, 1997; LeDoux, 1991).

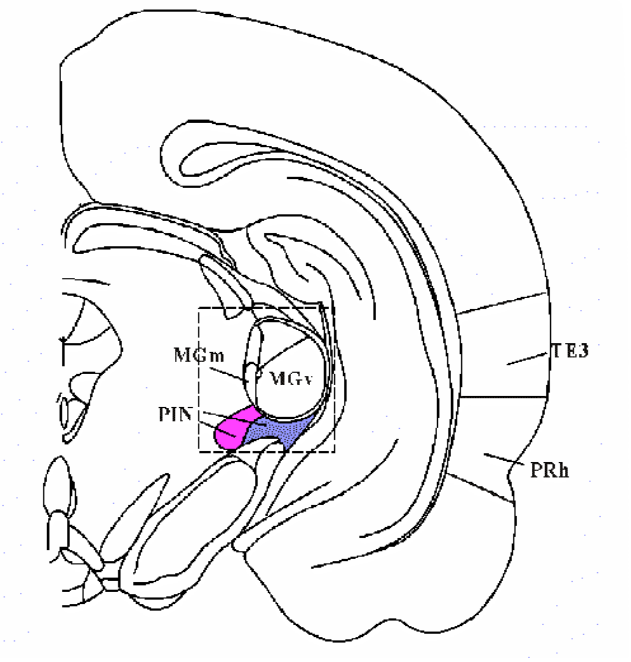

Figure 1. Schematic of the auditory thalamus. The figure shows the localization of the auditory thalamus; the MGM and PIN. In this schematic the PP and PIN have been combined into the PIN, however in the research that follows these structures are addressed separately. Therefore, for clarification the PP is depicted in blue, while the PIN is depicted in pink. The figure, approximately -5.3mm relative to bregma, is adapted from the atlas for the rat brain (Paxinos, 1997).

A role for the auditory thalamus in fear conditioning has been established previously. For example, lesions studies have revealed that it and its projections to the LA play a key role in auditory fear memory formation (Campeau, 1995; Jarrell, 1986; LeDoux, 1986; LeDoux, 1986). Subsequent physiological investigations have shown that fear conditioning changes neuronal excitatory levels in the auditory thalamus, in particular increased neuronal activity specifically tuned to the CS tone has been observed (Gabriel, 1975; Lennartz, 1992; McEchron, 1996). Recently, researchers have begun to turn their attention to molecular mechanisms within the auditory thalamus that underpin this plasticity and more importantly necessitate the formation of an auditory conditioned fear memory association. Microinfusions of mRNA synthesis inhibitors or ERK/MAPK inhibitors into the auditory thalamus impairs auditory fear memory consolidation (Apergis-Schoute, 2005; Parsons, 2006). Pre-training intra-thalamic microinfusions of the protein synthesis inhibitor anisomycin also produced a similar

deficit (Parsons, 2006). Finally, researchers using a gain of function approach have reported an enhancement of auditory conditioned fear in animals with increased levels of CREB in the auditory thalamus (Han, 2008).

It has been suggested that such intracellular signalling events within the auditory thalamus could serve to support the formation of an LA based fear memory representation, specifically in a presynaptic manner (Apergis-Schoute, 2005). In line with this, it seems probable that researchers highlighting the signalling events within the auditory thalamus necessary for fear memory formation could be examining components of signalling events that eventuate with presynaptic modifications within the LA. Indeed, there are a number of research findings in line with this. For example, blockade of ERK signalling in the auditory thalamus impairs LTP induced plasticity within the LA (Apergis-Schoute, 2005). Furthermore, post training intra-thalamic anisomycin microinfusions, as opposed to pre-training mentioned above, fail to impair auditory fear memory consolidation (Apergis-Schoute, 2005; Maren, 2003). As a result it was suggested that these post-training anisomycin infusions failed to produce an effect because at this time, protein synthesis could be occurring at synaptic terminals within the LA (Apergis-Schoute, 2005). Finally, both behavioural and electrophysiological studies have shown that conditioning or stimulation of thalamic inputs to the LA results in a long-term enhancement of presynaptic transmitter release at thalamo-LA synapses (McKernan, 1997; Zinebi, 2003). However, research concerning the precise intracellular signalling events or genomic modifications that lead to this presynaptic potentiation at LA-thalamo synapses is lacking.

As mentioned above, activation of the ERK/MAPK signalling pathway in the auditory thalamus is required for fear memory formation (Apergis-Schoute, 2005). One of the defining characteristics of the MAPK signalling cascade is its ability to modulate

genomic activity within a neuron. To this end, a logical question to ask is what genes are upregulated in the auditory thalamus as a result of fear conditioning induced MAPK regulation? The first wave of genomic activity after cellular excitation is an upregulation of IEG (Clayton, 2000). An IEG known to be downstream of MAPK signalling is EGR-1 (see below). Further, it is reliably associated with the protein synthesis-dependent phase of LTP where its upregulation is correlated with the persistence of LTP (Abraham, 1991; Abraham, 1993). Accordingly, at a behavioural level EGR-1 is known to play a role in the later phases of memory consolidation (Bozon, 2002; Davis, 2003; Davis, 2000; Jones, 2001).

The early growth response gene 1

EGR-1 (also known as Krox 24, NGFI-A, Zif268, TZS8 and Zenk) is one of 10-15 regulatory IEGs. EGR-1 contains a cyclic AMP response element (CRE) and serum response element (SRE) binding site. This means that it is regulated by the MAPK signalling cascade through the transcription factors CREB and ELK1, which bind to the CRE and SRE promoter region of EGR-1 respectively (Cruzalegui, 1999; Davis, 2000; Greenwood, 2002; Jannsen-Timmen, 1989; Sgambato, 1998; Whitmarsh, 1995). One distinguishing feature of EGR-1, and other EGR family members, is the 3 zinc finger sequences in the DNA binding domain of the protein that allows the protein to regulate the transcription of other genes by binding to the EGR consensus sequence (Christy, 1989; Swirnoff, 1995; Tsai-Morris, 1998). Specifically, once translated in the cytoplasm EGR-1 can enter the nucleus and regulate the transcription of target genes. Thus, given its upregulation after periods of cellular excitation, EGR-1 has been referred to as a nuclear intermediary in signal transduction, where is thought to

upregulate genes that could play a more direct role in cellular processes associated with memory formation.

The consequence or role of EGR-1 upregulation, or the upregulation of any IEG for that matter, during fear memory consolidation has, however, been debated. It has been suggested that gene upregulation 1) simply serves as a replenishment mechanism whereby levels of depleted cellular components are restored after periods of cellular excitation (Worley, 1991). An alternative interpretation is that IEG upregulation occurs to prime the cellular network for memory consolidation especially when an individual or animal is presented with a novel stimulus or situation, but does not necessarily assist directly in cellular processes associated with memory formation (Clayton, 2000; Tischmeyer, 1999). Finally, it is possible that EGR-1 upregulation serves to stabilize short-term cellular changes where it acts to upregulate proteins that are specifically tagged to recently activated synapse. Here EGR-1 based genomic modifications' are thought to support the newly acquired gain of function that occurred during STM formation, thus playing a more directly role in fear memory consolidation (Knapska, 2004). In an effort to delineate gene upregulation that occurs during memory formation from that which occurs as a result of stimulus novelty, substantial control groups are usually included within an experimental design. For example, with regard to fear conditioning, animals' are often presented with unpaired CS-US protocols, US alone, and CS alone stimulus presentations in a manner that is not conducive to learning. In such designs it is anticipated that there will be quantitative or qualitative differences in IEG regulation.

This current study

As discussed above, previous research has revealed that blockade of the MAPK signalling pathway or mRNA synthesis in the auditory thalamus prevents auditory fear memory consolidation (Apergis-Schoute, 2005). These results indicate that activation of intracellular pathways in the auditory thalamus are necessary for auditory conditioning. The first wave of gene response directed towards experience-dependent modifications of cellular functioning is the IEG response (Clayton, 2000; Tischmeyer, 1999). One relatively well characterized IEG is EGR-1, an inducible transcription factor that it thought to be necessary for the stabilization of both long-term synaptic plasticity and by relation LTM. It is though that its ability to upregulate effector genes could contribute to more enduring changes in synaptic efficacy necessary for memory consolidation (Bozon, 2002; Clayton, 2000). Indeed, a role for EGR-1 upregulation in the fear system has been reported previously as its regulation in the LA is required for fear memory consolidation (Ko, 2005; Malkani, 2004).

The aim of this first experiment was to determine whether auditory fear conditioning upregulates the expression of EGR-1 in a neuroanatomical region located presynaptically to the LA. Specifically within auditory thalamic cells within: the PP, PIN, MGM, and the ventral region of the medial geniculate (MGV). Rats were divided into 1 of 5 experimental conditions where EGR-1 upregulation associated with three tone-footshock pairings was compared to four other control conditions in which EGR-1 upregulation resulting from predisposing and concomitant factors in the absence of learning was observed; an immediate shock, unpaired tone and footshock, tone alone, and naïve group. A significant level of auditory thalamic EGR-1 upregulation in the Paired group compared to the other 4 groups would indicate that EGR-1 regulation as a function of this protocol could be solely attributed to learning associated gene

regulation. Furthermore, if the regulation occurs at a greater degree in thalamic structures with afferent projections to the amygdala, specifically all thalamic structures examined except for the MGV, the regulation may be related to presynaptic alterations that contribute to the formation of an amygdala based fear memory association.

Method

Subjects.

Twenty adult male Sprague Dawley rats (Hilltop Labs) were housed individually in plexiglass cages in a vivarium maintained on a 12 h light/dark cycle. All procedures were conducted during the light phase of the cycle. Food was available *ad libitum* throughout the experiment.

Conditioning Apparatus

The conditioning chambers were from Coulbourn Instruments (Whitehall, PA). They consisted of a standard grid floor conditioning chamber (Product # H10-11R-TC-SF; H10-11R-TC) contained within a noise and light attenuated box. Shocks were delivered through the grid floor using a standard animal shocker (Product # H13-15). Movement amplitudes were determined using a ceiling mounted infrared activity monitor and analysed using GS3 Graphic State notation 3 software. The same conditioning chambers were used for all experiments conducted for this dissertation.

Behavioural procedure.

The day before conditioning rats were habituated to handling for 5-10 min. The next day the animals were randomly divided into 1 of 5 groups; a Paired group, where rats were conditioned with 3 tone-shock pairings consisting of a 20 s, 5kHz, 75 dB tone that coterminated with a 1.0 s, 1.0 mA footshock presented through the grid floor of the

chamber. 2) An “Immediate Shock” group where animals were presented with 3 1.0 s, 1.0 mA footshocks. This protocol allows for examination of shock-induced gene upregulation in the absence of a learning associated shock-content contingency (Blanchard, 1976; Fanselow, 1980; Landeira-Fernandez, 2006). 3) An Unpaired group where the animals were presented with 3 immediate shocks as described above, followed by 3 tone presentations identical in characteristics to the tone used in the Paired group. 4) A Tone Alone group, where rats were presented with 3 tone presentations, again the tone was identical to that used in the Paired and Unpaired groups. 5) A Naïve group that were handled and killed without exposure to the conditioning chamber.

In each condition the animals were placed in a conditioning chamber and given 150 sec to acclimate, and the interstimulus interval (ISI) averaged 120 s, except for the immediate shock group where the stimuli were presented immediately upon the animals being placed in the chamber with an IS`I of 0.5 sec. The training time across all 5 behavioural conditions was 9 min.

Two hours after training, animals from all groups were euthanized with chlorohydrate (250 mg/kg, I.P. administration) and then transcardially perfused with ~50ml of phosphate buffered saline (PBS) followed by 250ml of ice cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The brains were promptly removed and postfixed in 4% paraformaldehyde-PB solution overnight then transferred into 20% glycerol-0.1 M PB cryoprotectant solution and stored at 4ºC until sectioning see Figure 2 for a schematic of the training protocol.

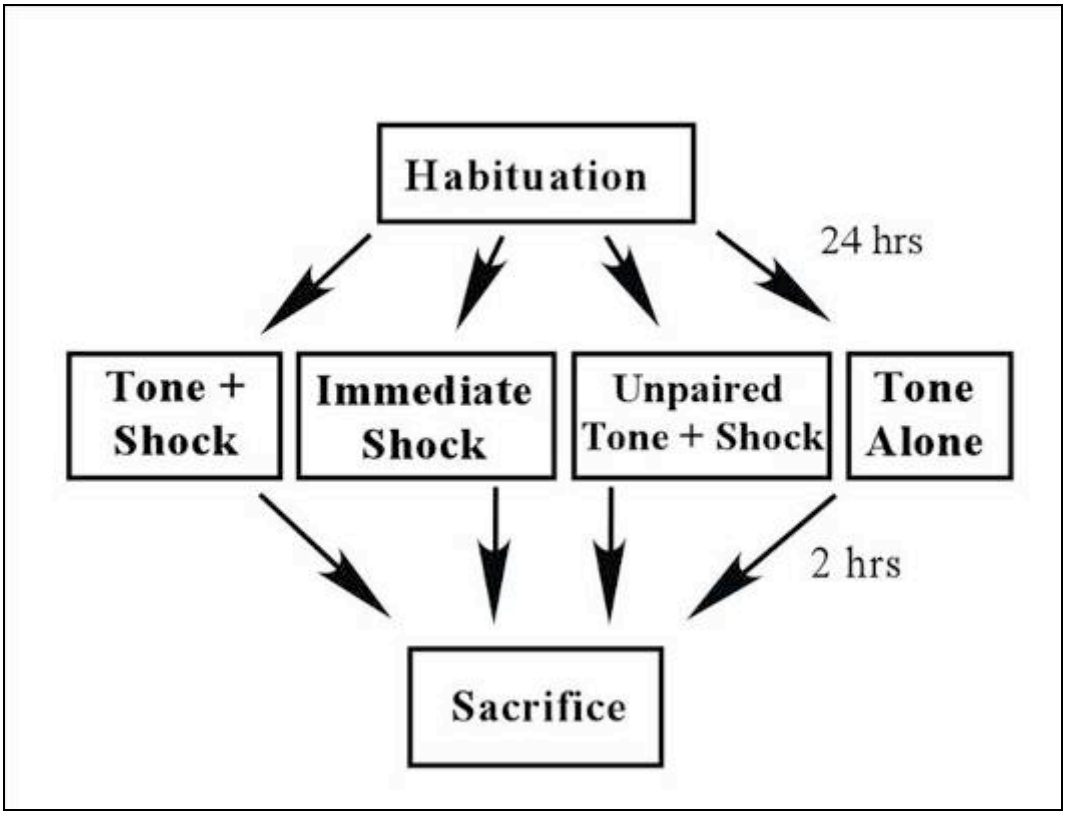

Figure 2. Training schedule for animals that were processed for the expression of EGR-1 immunoreactivity in the auditory thalamus as a function of conditioning (tone + shock), immediate shock, unpaired tone + shock and tone alone protocols. As depicted the animals were habituated to handling 24 hrs prior to training, 2 hrs post training animals were sacrificed and thalamic tissue processed for EGR-1 immunoreactivity.

Immunohistochemistry

Forty-micron free-floating sections of the auditory thalamus were taken using a sliding microtome and stored in a 1% sodium azide (NaAz) PBS solution until processing. Approximately 7 random thalamic sections from each animal were assayed for EGR-1 immunoreactivity. Sections were washed in PBS before being transferred to a blocking solution (1% albium from bovine serum (BSA; Sigma Fraction V, Cat # A-3059) and 0.1% Triton X-100 in PBS) for one hour. Sections were incubated overnight at room temperature in an anti-EGR-1 primary antibody (IgG-1) solution 1:2000 (ERG-1 Rabbit lot G-2304, catalogue number SC110), washed in PBS, and incubated for an hour in a secondary antibody solution (IgG-2) (biotin-conjugated goat anti-rabbit). The tissue was visualized using VectorStain ABC kit (Vector Laboratories) and developed

in a 3,3'-diaminobenzidine (DAB) peroxidase substrate for approximately 7 min. Finally, sections were washed and mounted on Fisherbrand electrostatics slides and cover slipped for later viewing. (Note: in the research mentioned in the following chapters EGR-1 immunoreactivity was assayed in exactly the same manner as described above).

Data Analysis

Thalamic sections between -5.3 and -6.04 relative to bregma were selected for scoring. Cell counts were taken from approximately three sections per animal, and scored using a defined boundary around the PP, PIN, MGM or MGV using ImageJ (Media Cybernetics, Silver Spring, MD). For analysis, cell counts across the left and right hemispheres were averaged to give a single score for each animal, group means were then determined and analyzed using analysis of variance (ANOVA) and *t*-test where appropriate. Dunnett's *t* was the specific *t*-test of choice as it allows explicit comparison of the Paired group against all controls (Note: analysis of EGR-1 immunoreactivity in the following Chapters was conducted as described above).

Results

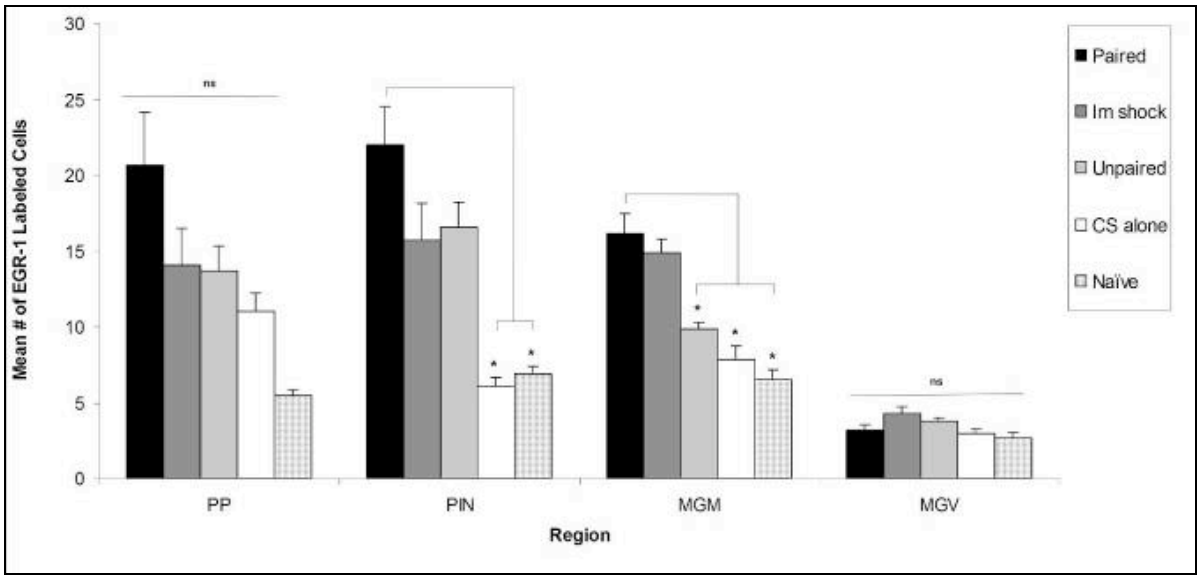

Figure 3. Regional breakdown of EGR-1 mean cell counts (+SEM) within structures of the posterior thalamus as a function of training. All rats were handled and then received either 3 tone shock pairings, 3 immediate shocks, 3 unpaired tone and shock presentations, 3 tone only presentations, or returned to their home cage; naïve. All animals were sacrificed 2 hrs after training (n=4 in each group). *p<0.05

Figure 3 shows the average EGR-1 upregulation across 4 structures within the posterior thalamus as a function of 5 different training protocols. The results indicated that EGR-1 upregulation was greater in the Paired group compared to all other training protocols across the 3 thalamic structures that project to the LA; the PP, PIN, and MGM. A one-way ANOVA revealed a significant difference across groups in the PIN (F(4,15)=3.76,p<0.03) with the Paired group differing significantly from the CS-Alone and Naïve animals (p<0.05 Dunnett's t), and MGM (F(4,15)=5.86,p<0.006) with the Paired group differing significantly from the Un-Paired, CS-alone, and Naïve animals (p<0.05 Dunnett's t). Despite a clear trend where the Paired group displayed a greater degree of EGR-1 upregulation compared to all control groups a one-way ANOVA failed to reach significance across the PP (F(4,15)=1.67,p>0.2). No significant differences were observed in the MGV (p>0.05).

To further analyze training induced regulation of EGR-1, cell counts were collapsed across the three thalamic structures that project to the LA into one average

score, allowing us to examine average EGR-1 counts across the auditory thalamus in its entirety. The results of this analysis are shown in Figure 4.

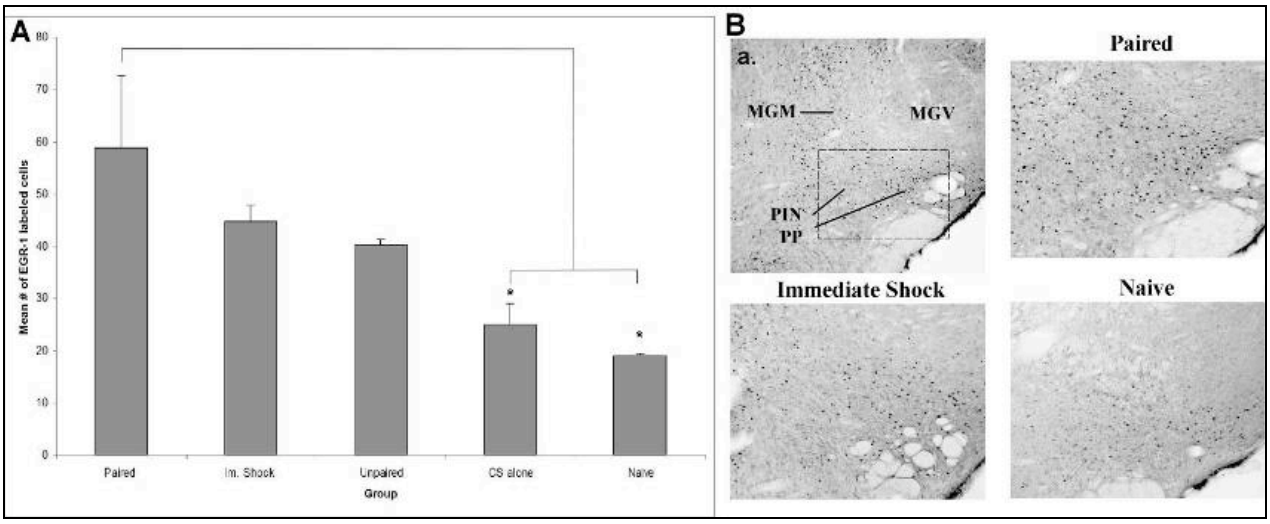

Figure 4. **A.** Training induced mean EGR-1 upregulation (+SEM) in the MGM, PIN, and PP combined. All rats were handled and then received either 3 tone shock pairings, 3 immediate shocks, unpaired tone and shock presentations, 3 tone only presentations, or returned to their home cage; naïve, and were sacrificed 2 hours after training (n=4 in each group). *p<0.05. **B.** Schematic representations' of EGR-1 immunoreactivity in the auditory thalamus. **a.** Example of the auditory thalamus depicting the localization of the 4 thalamic nuclei processed. Representative immunoblots from a Paired, Immediate Shock and Naïve animal are shown at a higher magnification from the region highlighted in **a.**

The results indicate that EGR-1 upregulation was greatest in the Paired group compared to the other 4 control conditions. A one way between groups ANOVA proved to be significant (F(4, 15)=5.90,p<0.006), with the Paired group differing significantly from both the CS alone and Naïve group (p<0.05; Dunnett's t).

Discussion

The aim of this study was to examine training induced EGR-1 upregulation in auditory thalamic cells. EGR-1 is an inducible IEG known to play a role in memory consolidation, (Bozon, 2002; Davis, 2003; Knapska, 2004). Immunohistochemistry was used to analyze EGR-1 upregulation in thalamic tissue thus allowing us to quantify regulatory differences at a nuclei level. However, in the series of experiments that constitute this dissertation specificity of immunostaining for EGR-1 was not examined.

Nonetheless, research results from our lab where westernblotting and *in situ* hybridization were used to detect EGR-1 protein and mRNA levels respectively have demonstrated the same pattern of EGR-1 upregulation in training paradigms. This suggests that the immunoreactivity observed in the thalamic sections was indicative of EGR-1 staining. However, a pre-absorption study would have confirmed this. Furthermore, a comparison of EGR-1 upregulation between the three nuclei that comprise the auditory thalamus would have been interesting. More specifically, if an antibody for a neuronal marker such as NeuN had been included with the EGR-1 incubation then the number of neurons μm^2 within each structure could have been determined. As a result the percentage of cells within each structure that contained training induced EGR-1 could have been determined allowing comparisons between nuclei and subsequent experiments conducted below. Nonetheless, combining the PP, PIN, and MGM into the :"auditory thalamus" allowed for a simple but yet but by no means less informative analysis of conditioning induced EGR-1 expression.

Previous research has indicated that the upregulation of IEGs often correlates with novel sensory stimulation (Tischmeyer, 1999). To this end, a comparison was made between EGR-1 regulation in paired animals to four control groups where stimuli were presented in such a manner that gene regulation as a function of predisposing and concomitant factors was observed. The results indicated that presentation of a temporally paired tone-footshock stimulus resulted in a greater degree of EGR-1 regulation when compared to the tone alone and naïve animals. As far as gene upregulation as a function of stimulus novelty is concerned, a difference was not observed between the Immediate Shock and Unpaired groups (data not shown). If EGR-1 gene upregulation was simply a function of sensory stimulation, or novelty, the unpaired protocol group should exhibit a greater degree of EGR-1 regulation compared

to the Tone-Alone group as they were presented with a greater degree of novel stimuli. The immediate shock and unpaired tone-foot-shock presentations, while producing a lower level of EGR-1 upregulation compared to the paired animals, did not prove to differ significantly from animals who received three CS-US conditioning trials. However, these groups did not differ statistically from the other controls (data not shown), indicating that this EGR-1 upregulation could of occurred irrespectively of any learning.

EGR-1 upregulation in conditions that do not support learning have been reported previously. For example, Malkani and Rosen (2000) compared contextual conditioning induced EGR-1 upregulation to 3 control groups 1) Naïve, 2) context-no-shock, and 3) immediate-shock. In this experiment EGR-1 expression was significantly greater in the immediate shock group compared to the other controls. However, the paired animals exhibited a significantly greater degree of EGR-1 upregulation compared to all other groups. In a similar study, Hall (2000) compared EGR-1 upregulation in contextual conditioned animals, naive controls, and latent inhibition animals. The latent inhibition group were exposed to the conditioning environment a day prior to contextual conditioning. This previous exposure to a CS-stimulus impairs subsequent conditioning to this CS. Using this protocol an equivalent level of EGR-1 upregulation was observed in both the context conditioned animals and the latent inhibition group. However, both groups significantly differed from the context only animals. That is, both Hall (2000) and Malkani (2000) reported a significant level of EGR-1 upregulation in training protocols that do not support the formation of a context-footshock association. As a result, the gene regulation observed in these two studies and in the immediate shock and unpaired control groups employed in this current study could be indicative of a priming mechanism whereby exposure to a novel or stressful

stimulus enhances storage efficacy of cells via gene upregulation that serves as a necessary but not sufficient precondition for memory consolidation (Clayton, 2000; Grimm, 1997). Indeed, footshock presentations are arousing in nature (Davis, 1989), and could consequently orientate the animal toward a readied state for memory formation, which, however, in these circumstances most probably failed to eventuate in a salient memory representation.

Another interpretation concerning the results of this experiment is that the homogeneity of means in the Paired, Immediate Shock, and Un-Paired groups may be a function of the inability to distinguish between thalamic cells that specifically project to the LA and thalamic afferents that terminate in other brain areas. The premise for this experiment was the hypothesis that activation of intracellular pathways within the auditory thalamus could occur to support the formation of an LA based fear memory representation. Anatomical localization of thalamic LA-projection cells, specifically those that can be considered a component of a LA-based circuitry, and analysis of EGR-1 regulation within this subset would result in a more unambiguous analysis of gene regulation within a more specific amygdala based fear network. Analysis of such gene regulation would most probably result in more substantial differences training induced EGR-1 upregulation between Paired and Control groups.

In this study the results have shown that training induces EGR-1 upregulation in the auditory thalamus. Given the fact that plasticity within the auditory thalamus is necessary for the formation of an auditory fear memory association while not being sufficient for its formation (Schafe, 2005), it is assumed that this genomic upregulation serves to support an LA-based fear memory representation. A significantly greater degree of EGR-1 upregulation in the Paired animals when compared to the Immediate Shock and Unpaired groups was not observed. However, the results obtained in this

research are confounded with a caveat whereby in this analysis it was impossible to specifically identify cells within the auditory thalamus that project to the LA and thus constitute the thalamo-LA synaptic connection of interest. As stated, the underlying premise for this experiment is that the LA is the key neuroanatomical locus of a fear memory association. If this is indeed the case then it is likely that LA-based signalling is required for the EGR-1 upregulation observed in the above experiment.

Chapter 3

A. Cellular excitation and Nitric oxide synthesis in the Amygdala regulates training induced EGR-1 upregulation in the Thalamus

Amygdala based modulation of gene expression in fear associated neuroanatomical areas

There is a great deal of anatomical, molecular, and electrophysiological research suggesting that the LA is a key neuroanatomical locus of conditioned fear (For example see Huff, 2004; Schafe, 2005). As well as being a key CS-US association area, the LA is thought to regulate intracellular activity associated with memory storage in other brain areas (Chavez, 2008; Huff, 2004, 2004; McGaugh, 2002; McIntyre, 2003). For example, pre-training intra-LA microinfusions of the $GABA_A$ receptor agonist muscimol prevents contextual fear conditioning induced increases in hippocampal Arc- and cFos-mRNA assessed 60 minutes post training (Huff, 2006). In a similar manner, McIntyre and colleagues (2005) have also reported LA-based modulation of hippocampal Arc expression during consolidation of an inhibitory avoidance memory. In this study, they infused a β- adrenergic receptor agonist into the LA immediately after training and reported a significant increase in dorsal hippocampal Arc protein levels 45 minutes later. Behaviourally, this was accompanied by a significant increase in task performance assessed 48 hours post-training. However, their reported increase in Arc protein was not accompanied by an increase in Arc mRNA, which contradicts the abovementioned findings of Huff and colleagues (2006). There were some methodological differences that could account for the difference of results, specifically, different conditioning paradigms, pre versus post drug infusions, differing shock intensities, different sacrifice time points, and manipulation of different molecular systems within the LA. Nonetheless, both these research findings illustrate the fact that the LA modulates training-induced increases in IEG expression in the hippocampus.

Furthermore, based on their results McIntyre and colleagues (2005) suggested that the LA is capable of modulating synaptic plasticity in efferent brain regions in a posttranslational manner (McIntyre, 2005). However, this LA based modulation and that reported by Huff and colleagues (2006) could be occurring in a retrograde type manner whereby the LA modulates afferent inputs from the hippocampus via a retrograde signalling molecule. A likely candidate could be the retrograde transmitter NO.

Evidence for retrograde signalling in the fear system

Research has shown that LA-based NO signalling is required for fear memory consolidation (Chein, 2008; Ota, 2008; Overeem, 2006; Schafe, 2005a). Nitric oxide is a signalling molecule that diffuses in an isotropic manner, thus giving it the capability of modulating cellular activity in presynaptic cells. Furthermore, NO signalling is known to increase the extracellular levels of norepinephrine (NE) (Jones, 1994, 1995; Lauth, 1995; Stout, 1994, 1995), by directly interacting with the NE transporter (Kaye, 2000; Lonart, 1992). Thus, NO may be upstream of, or at least contribute to, NE induced modulation of IEG expression in the hippocampus (McIntyre, 2005). As mentioned above, the authors attributed the increase in Arc protein expression to posttranslational modulation of gene expression by the LA. There is evidence that NO can, in fact, modulate stability of mRNA at a posttranscriptional level (Johnston, 1994a, 1994b; Morris, 1997). Thus, it is possible that retrograde signalling could account for amygdaloid modulation of gene expression in afferent fear related processing areas. A key question for my research is whether this is occurring at thalamo-LA synapses?

Neuronal-nitric-oxide-synthase (nNOS) colocalizes with a NMDA receptor via the post synaptic density protein PSD-95 (Christopherson, 1999; Garner, 2000;

Kennedy, 1993; Kornau, 1995; Sattler, 1999; Ziff, 1997). NMDA receptors have been localized at excitatory auditory thalamo-LA synapses (Bordi, 1994; Farb, 1997; J. E. LeDoux, & Farb, C.R., 1991; Li, 1995; Weisskopf, 1999). In line with this, nNOS and has also been localized at excitatory synapses within the LA (McDonald, 1993; Schafe, 2005; Unger, 1992). Activation of LA based NMDA receptors is required for fear memory consolidation (Bauer, 2002; Campeau, 1992; Kim, 1991; Rodrigues, 2001; Walker, 2000). Neuronal-NOS is indirectly activated via the calcium influx through activated NMDA receptors. Thus, the anatomical, or more specifically molecular, construction of thalamo-LA synapses appears to be able to support retrograde signalling processes.

Nitric oxide as a modulator of cellular processes associated with gene expression

The ability of NO, its metabolites, or the downstream kinase PKG, to modulate processes associated with gene expression is well documented. Firstly, regulation can occur at an extracellular level where NO can facilitate cellular excitation by interacting with the GABAergic and glutamatergic neutransmitter systems. NO can reduce chloride currents that pass through the $GABA_A$ receptor in a PKG dependent manner (Robello, 1996; Wall, 2003; Wexler, 1998; Zarri, 1994), and facilitate glutamatergic extracellular excitation by directly interacting with vesicle docking proteins (Meffert, 1996, 1994; Ohkuma, 2001; Segovia, 1994). With regard to modulatory neurotransmitters, as mentioned above, it can increase the extracellular concentration of NE. It also interferes with dopamine (DA) reuptake by impeding the function of reuptake transporters, consequently increasing its extracellular concentration (Kiss, 2000, 2001, 2004). Secondly, NO modulation of gene expression can also occur at both an intracellular signalling and genomic level. NO can activate the MAPK pathway by directly

interacting with Ras, the small GTPase directly upstream of the cascade (Lander, 1996, 1995; Yun, 1998). It can also indirectly activate components of the MAPK cascade via PKG (Endo, 2003; Ota, 2008; Yamazaki, 2005). Further, PKG activity has been reported downstream of MAPK at a transcriptional level, where it has been reported to contribute to CREB phosphorylation (Chein, 2008; Lu, 1999). Thirdly, as mentioned above, NO is capable of acting at a posttranscriptional level by stabilizing mRNA levels (Johnston, 1994a, 1994b). Given NO's ability to act as a retrograde neurotransmitter and regulatory influence on numerous cellular processes associated with genomic modifications, if the LA is indeed regulating cellular signalling within the auditory thalamus, NO may be mediating this activity.

In line with this, LA excitation is necessary for the development of conditioned induced plasticity in the auditory thalamus (Maren, 2001; Poremba, 2001). Given that EGR-1 upregulation has been linked to synaptic plasticity (Abraham, 1993; Cole, 1989; Richardson, 1992) it stands to reason that the LA could be modulating training-induced EGR-1 upregulation in the auditory thalamus. Indeed, NO has been linked to EGR-1 upregulation via or in association with the MAPK cascade (Chiu, 1999; Cibelli, 2002; Esteve, 2001; Hemish, 2003; Morris, 1995). Activation of the MAPK pathway in the auditory thalamus is also a prerequisite for fear memory consolidation (Apergis-Schoute, 2005). This research finding and the above discussion concerning NO ability to modulate cellular process associated with genomic modifications collectively point to the notion that NO signalling may be upstream of training induced EGR-1 upregulation in the auditory thalamus.

Research has suggested that LA modulation of plasticity related processes occurs during the acquisition phase of memory formation (Poremba, 2001). Moreover, molecular based research has shown that intra LA microinfusions of the ERK/MAPK

inhibitor U0126 do not affect training induced conditioned induced thalamic plasticity (Schafe, 2005). Given the spatial relationship of nNOS with the NMDA receptor, and the receptors' pivotal role in the acquisition of a fear memory association, it stands to reason that if NO is indeed, regulating EGR-1 expression in the auditory thalamus it is doing so during the acquisition phase of fear memory formation.

This current study

In the following series of experiments various pharmacological compounds were microinfused into the LA and an examination of the effect this had on training induced EGR-1 upregulation in the auditory thalamus was done. Firstly, intra-LA microinfusions of the $GABA_A$ receptor agonist muscimol were employed to determine whether LA cellular excitation is required for thalamic EGR-1 upregulation. Next, the selective nNOS inhibitor 7-ni and the membrane impermeable NO scavenger 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) were used to determine whether LA based NO synthesis is required for EGR-1 upregulation. Finally, an experiment was conducted to test whether intra-LA microinfusions of the ERK kinase inhibitor U0126 affected conditioned induced EGR-1, thus examining the time frame during which NO signalling affects thalamic gene upregulation. If the LA is indeed modulating EGR-1 upregulation in the auditory thalamus via the retrograde signalling molecule NO it is expected that a significant reduction in EGR-1 expression in animals who receive $GABA_A$ agonist and nNOS antagonists or NO scavengers. If the LA is modulating EGR-1 expression during the acquisition phase or fear memory formation it is anticipated that the U0126 microinfusions will not affect training induced EGR-1 expression in the auditory thalamus.

Method

Subjects

Twenty-five adult (300-350g) male Sprague Dawley Rats (Hilltop Laboratories, Philadelphia, PA) served as subjects. They were housed individually in plastic cages with free access to food and water. The colony was maintained on a 12 hr light dark cycle.

Surgery

Rats were anethnatised with a ketamine (95mg/Kg) and xylazine (0.05mg/kg) mixed solution, mounted in a sterotaxic and implanted with guide cannulas (26 gauge; Plastics One, Roanoke, VA) aimed at the LA (AP: -3.3, ML: ±5.0, DV: -8.0). The implants were secured to the skull with acrylic cement and jeweller screws and dummy cannulae inserted. Immediately after surgery animals received an IP administration of buprenex (0.5ml 0.03mg/ml). Three days following surgery, ibuprofen was freely available to the animals in their water supply.

Drugs

The $GABA_A$ receptor agonist muscimol (0.25µg; 0.5µl) and the NO scavenger cPTIO (1.0µg; 0.5µl) were dissolved in physiological saline. The MEK inhibitor U0126 was dissolved in dimethyl sulfoxide (DMSO) to produce a stock solution of 2µg/µl. Before infusion the drug was diluted with artificial cerebral spinal fluid (ACSF) 1:1. The global NOS inhibitor 7ni was dissolved in ACSF. The above drugs or vehicle solutions were bilaterally infused into the LA at a rate of 0.2µl/min.

Behavioural procedures

Two days prior to training the animals were handheld and habituated to dummy cannulae removal. The following day the animals were handheld and the dummy cannulae removed. Infusion cannulas that projected 1mm below the tip of the guide

were inserted and the appropriate drug infused. Once infusion was complete, the infusion cannulae were retained in the guide for a further 1 minute to allow drug diffusion from the tip.

Behavioural training commenced thirty minutes after drug infusion. Rats were placed in the conditioning chambers and allowed 150 seconds to acclimate. They were then conditioned in the same manner as the Paired animals described in Chapter 2. More specifically, they were presented with three tone light shock pairings at an interstimulus interval (ISI) of 120 sec. The tone was presented for 20 seconds and coterminated with a 1 second 1 mA footshock. Total time in the conditioning chamber was approximately 9 min.

Perfusions

Two hours after training, rats were euthanized with 1ml of chlorohydrate (250 mg/kg, I.P. administration). They were immediately transcardially perfused with ~50ml of phosphate buffered saline (PBS) followed by 250ml of 4% paraformaldehyde. The brains were removed and postfixed in 4% paraformaldehyde solution overnight then transferred to a 20% glycerin cryoprotectant solution until sectioning. See Figure 5. for a schematic of the training protocol used.

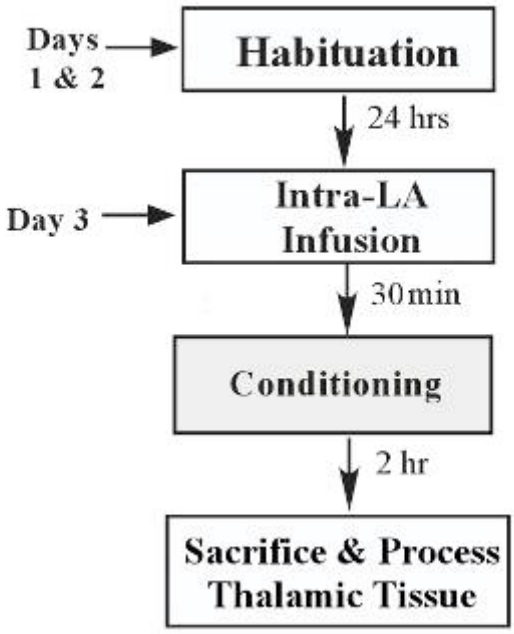

Figure 5. Schematic of the training protocol used for analysis of training induced EGR-1 upregulation as a function of intra-LA microinfusions of muscimol, cPTIO, U0126, 7ni, and ifenprodil. Animals were habituated to handling and cannulae removal on two consecutive days prior to conditioning. Thirty minutes prior to conditioning they received intra-LA drug infusions. Two hours post training they were sacrificed for processing of thalamic tissue for EGR-1 immunoreactivity. .

Histology

Forty-micron free-floating sections of the amygdala and auditory thalamus were taken using a sliding microtome and stored in a 1% sodium azide solution (NaAz) in PBS. Amygdaloidal sections were mounted on electrostatic slides, stained using cresyl-violet, coversliped, and cannula placements verified. Thalamic sections were assayed for EGR-1 protein expression.

Immunohistochemistry

Immunohistochemistry protocols were identical to those used in the research conducted in Chapter 2.

Data Analysis

EGR-1 counts were analysed in exactly the same manner as described in Chapter 2.

During training behavioural freezing prior to the 1 sec shock presentation and 20 sec post shock presentation were recorded. Total seconds of freezing during each time period were recorded and expressed as a percentage of behavioural freezing. Results were compared using ANOVAs and *post hoc* Dunnett's *t*-tests were appropriate.

Results

LA cellular excitation and NO signalling from the LA are required for the expression of EGR-1 in the auditory thalamus

Histology

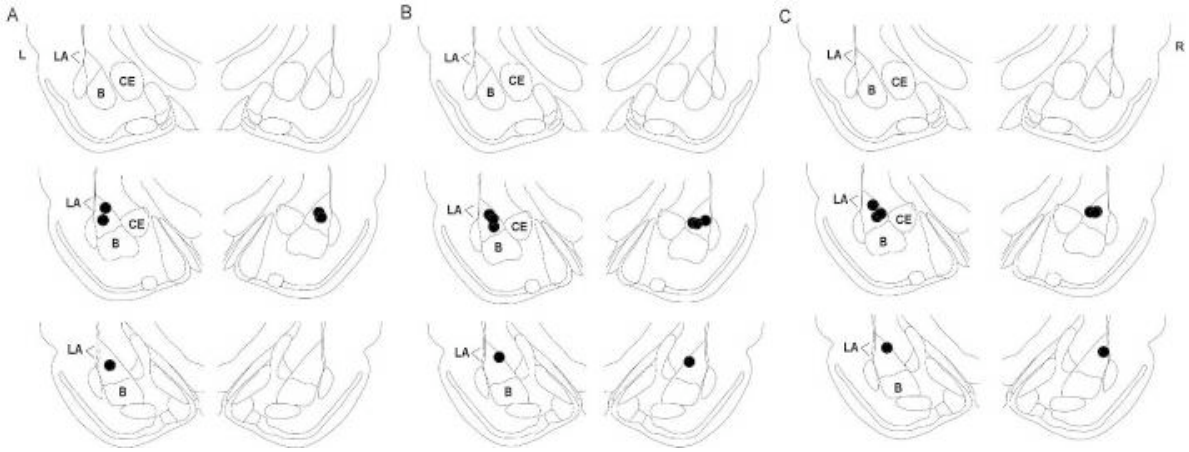

Figure 6. Schematic coronal sections depicting the intra-LA cannula locations for A) Vehicle, B) Muscimol, and C) cPTIO animals. LA: lateral amygdala, B: Basal amygdala, CE, Central nucleus of the amygdala

Figure 6 depicts the guide cannula placements for animals that received Vehicle, Muscimol or cPTIO microinfusions. All placements are superimposed on coronal sections taken from the rat brain atlas (Paxinos, 2004). As depicted, the cannula tips were predominantly located in the medial portion of the LA.

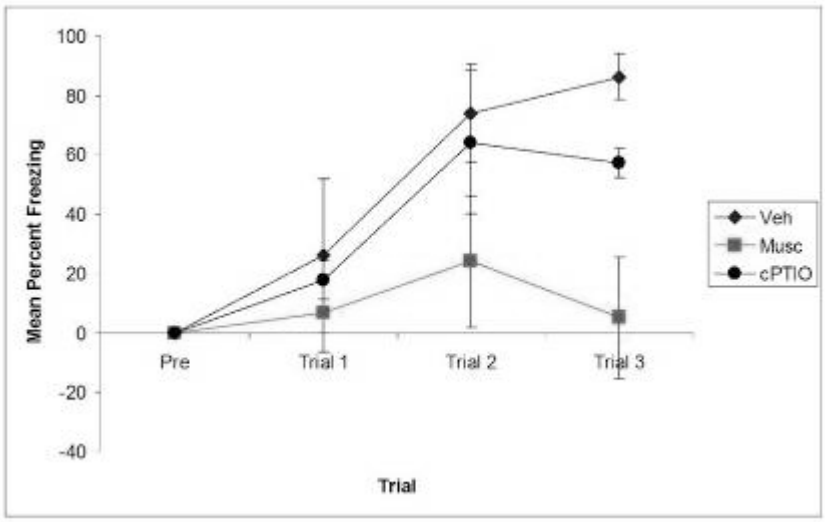

Figure 7. Average percent freezing (± SEM) pre and post shock across the three conditioning trials as a function of intra LA microinfusions of muscimol (n=4), cPTIO (n=4) and vehicle (n=3).

Figure 7 shows the average percent freezing for Vehicle, Muscimol, and cPTIO groups prior and during conditioning. Post shock freezing appears to be intact for both the Vehicle and Muscimol groups. A one-way ANOVA comparing each group at each of the trials failed to reach significance at trial one ($F(2,8)=0.410, p>0.6$) and trial two ($F(2,8)=1.427, p>0.2$), but proved to be significant at trial three ($F(2,8)=8.65, p<0.01$) where only the Muscimol group differed significantly from the vehicle controls (Dunnett's *t*, $p<0.007$). This is expected, given the inhibitory activity muscimol has on US-associated processing (Van Nobelen, & Kokkindis 2006). Overall, the results show that both Vehicle and cPTIO microinfusions failed to impair the animals' perception of the footshock-US.

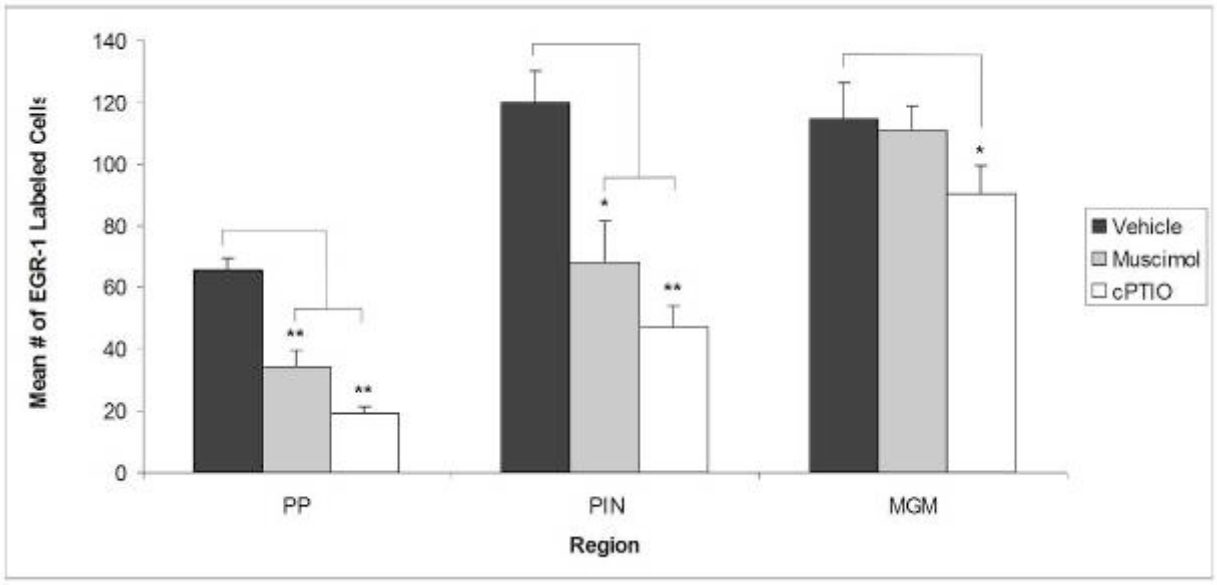

Figure 8. Average EGR-1 cell counts (±SEM) in the PP, PIN, and MGM nuclei of the thalamus for groups that received microinfusions of saline (n=3) muscimol (n=4), or cPTIO (n=4), into the LA. Microinfusions occurred 30 minutes prior to conditioning. Animals were sacrificed 2 hours post training. * $p<0.05$, ** $p<0.001$

Figure 8 shows the EGR-1 protein levels across the PP, PIN, and MGM of the auditory thalamus as a function of intra-LA vehicle, muscimol, or cPTIO microinfusions. EGR-1 counts in the PP were less for animals that received muscimol or cPTIO when compared to vehicle controls. A one-way ANOVA revealed a significant result ($F(2,8)=29.96, p<0.001$), with both the Muscimol and cPTIO groups differing significantly from vehicle controls (Dunnett's t, $p<0.001$ in both cases). Within the PIN a similar result was observed, again a one-way ANOVA revealed a significant effect ($F(2,8)=11.037, p<0.005$), with further analysis using Dunnett's t showing that both the muscimol ($p<0.02$) and cPTIO ($p<0.003$) groups exhibited significantly lower EGR-1 counts when compared to vehicle controls. Finally, analysis of EGR-1 counts within the MGM also produced significant results (one way ANOVA: $F(2,8)=4.655, p<0.05$) with only the cPTIO group differing significantly from vehicle controls (Dunnett's t, $p<0.03$).

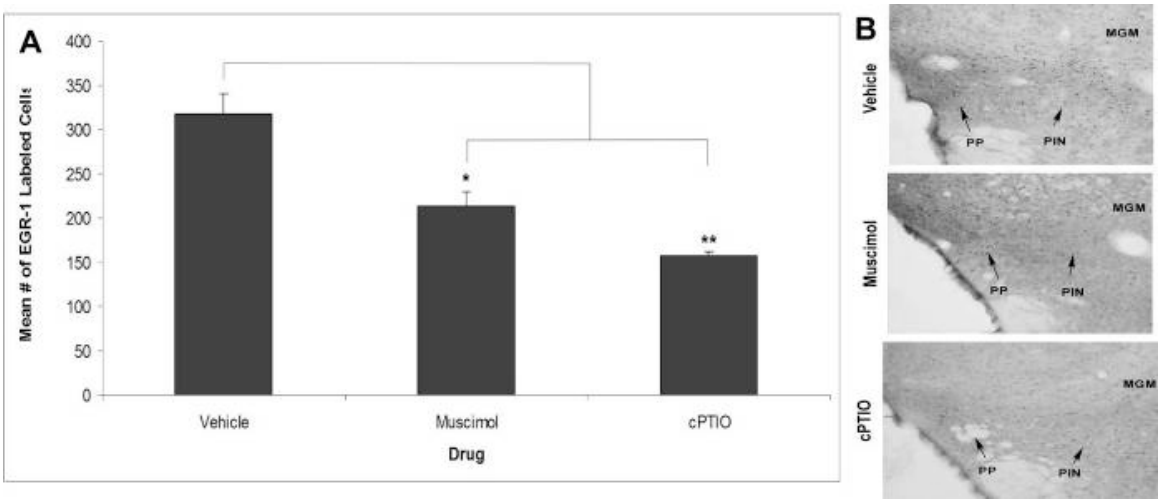

Figure 9. **A.** Average EGR-1 cell counts (±SEM) across the auditory thalamus (PP, PIN, and MGM combined) for groups that received microinfusions of vehicle (n=3) muscimol (n=4), or cPTIO (n=4), into the LA 30min prior to fear conditioning. EGR-1 protein levels were assessed 2 hours post conditioning. * $p<0.05$, ** $p<0.001$. **B.** Representative micrographs of EGR-1 immunoreactivity in the auditory thalamus.

For further analysis the nuclei of the auditory thalamus were combined and the effect of intra-LA muscimol and cPTIO microinfusions upon EGR-1 upregulation of the auditory thalamus as a whole was examined (see Figure 9). A one-way ANOVA revealed a significant effect, $F(2,8) = 25.314, p<0.001$, with both the Muscimol and cPTIO groups exhibiting a significantly lower EGR-1 count when compared to vehicle controls (Dunnett's t for both $p<0.003$). Overall, the results show that both LA cellular excitation and NO diffusion into the extracellular space is required for conditioning induced increases in EGR-1 protein levels in the auditory thalamus.

EGR-1 upregulation in the auditory thalamus is dependent on LA neuronal-nitric oxide-synthase-activation but not LA ERK signalling

Histology

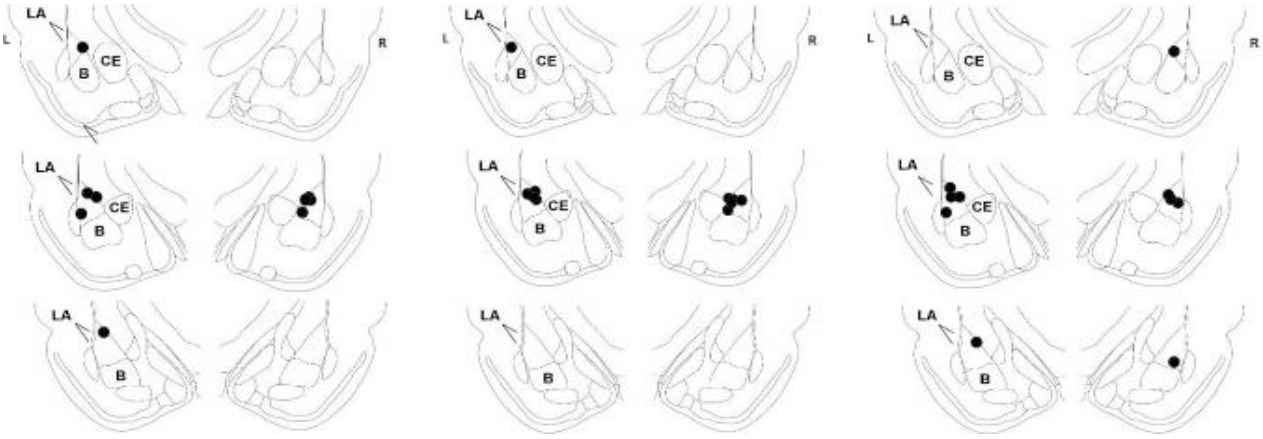

Figure 10. Schematic coronal sections depicting the intra-LA cannula locations for A) U0126, B) 7-NI, and C) vehicle control animals. LA: lateral amygdala, B: Basal amygdala, CE, Central nucleus of the amygdala.

Figure 10 shows the cannula tips for animals that received U0126, 7-ni, and vehicle microinfusions. All placements are superimposed on coronal sections taken from the rat brain atlas (Paxinos, 2004). As illustrated, the cannula placements were predominantly in the medial portion of the LA.

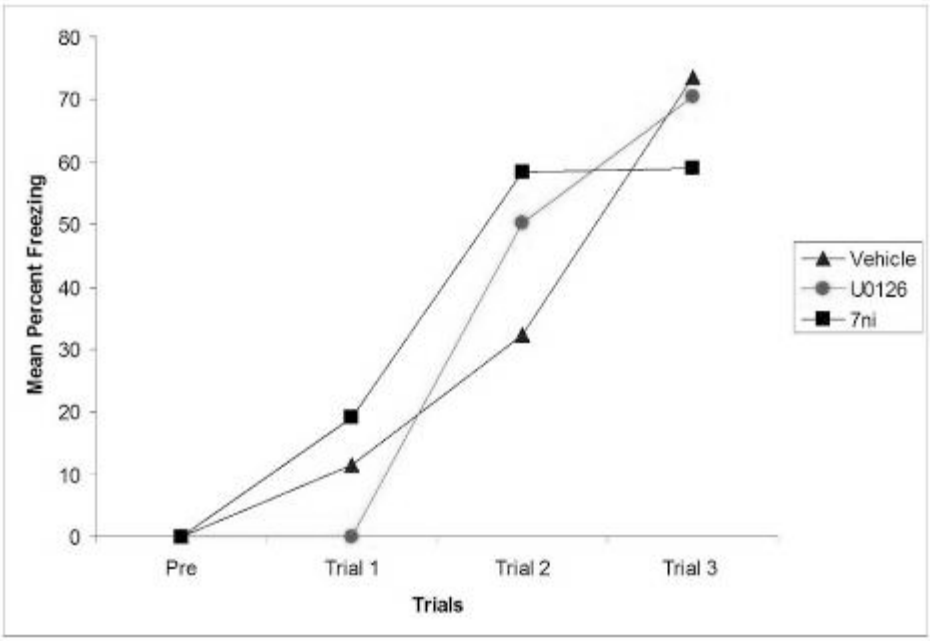

Figure 11. Average percent freezing (± SEM) pre and post shock across the three conditioning trials as a function of intra LA microinfusions of ACSF-vehicle (n=5), U0126 (n=4) and 7ni (n=5).

Figure 11 shows average movement amplitudes prior to conditioning and postshock freezing for animals who received intra-LA microinfusions of ACSF-Vehicle, U0126, and 7ni. The figure shows that all three groups exhibited similar levels of postshock freezing across the three conditioning trials. For each of the three conditioning trials a one way ANOVA was performed comparing the average freezing for each group. As expected, no significant differences were observed (for each conditioning trial $p>0.60$), indicating equivalent levels of postshock freezing across all groups at each training trial. Thus, it is reasonable to conclude that all drug infusions failed to impair the animals' perception of the US.

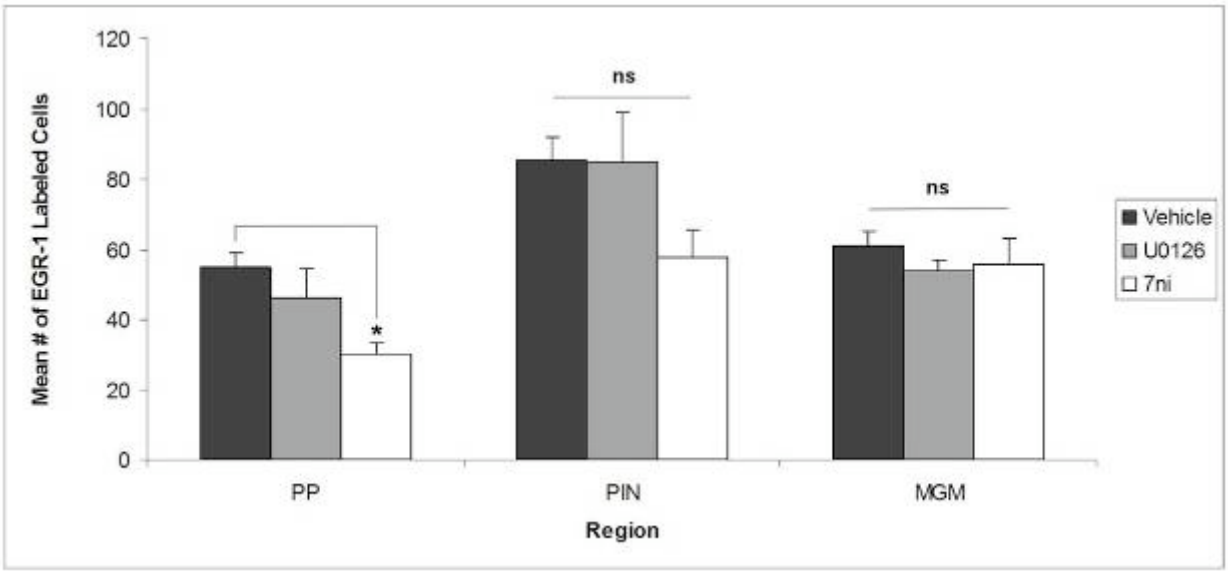

Figure 12. Average EGR-1 cell counts (±SEM) in the PP, PIN, and MGM nuclei of the thalamus for groups that received microinfusions of ACSF-vehicle (n=5) U0126 (n=4), or 7ni (n=5), into the LA. Animals were sacrificed 2 hrs post training. * $p<0.05$

Figure 12 shows the average EGR-1 count across the PP, PIN, and MGM of the thalamus. Across both the PP and PIN the 7ni group appeared to exhibit substantially EGR-1 immunoreactivity compared to the vehicle-control animals. One way ANOVAs' revealed a significant effect within the PP ($F(2,11)=6.54, p<0.02$), but, while suggestive, failed to reach significance in the PIN ($F(2,11)=3.06, p>0.08$). As anticipated, given the similarities across counts observed in the MGM, no significant findings were observed ($F(2,11)=0.422$, $p>0.6$).

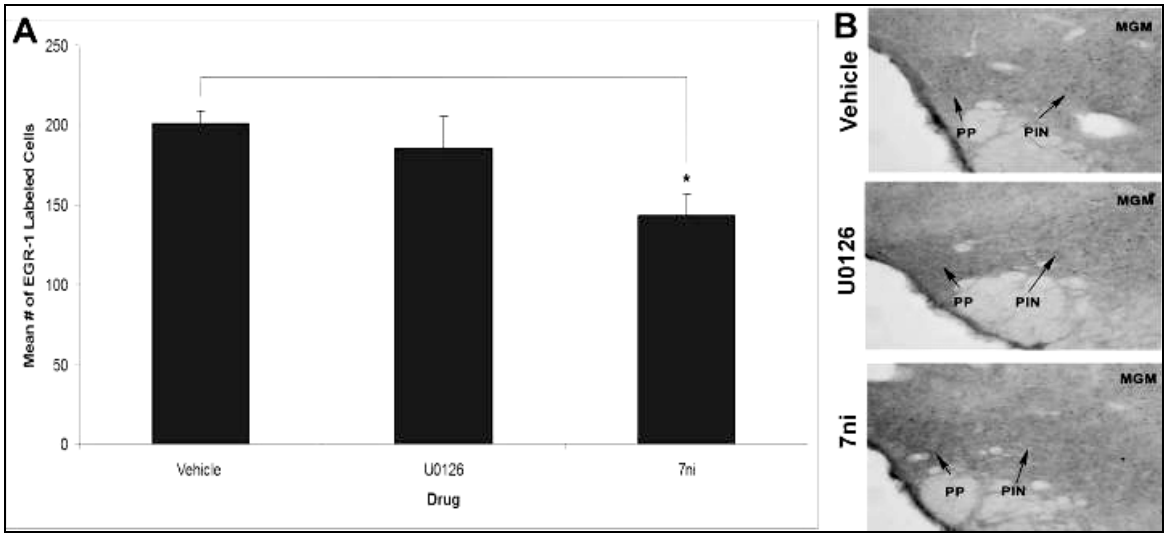

Figure 13. **A.** Average (± SEM) EGR-1 cell counts in the auditory thalamus (PP, PIN, and MGM combined) after miroinfusion of U0126 (n=5) 7ni (n=5) or ACSF-vehicle (n=5) into the LA thirty minutes prior to fear conditioning. Animals were sacrificed 2 hours post conditioning. * p <0.05. **B** Representative micrographs of EGR-1 immunoreactivity in the auditory thalamus.

Figure 13 shows the affect of intra-LA microinfusions of U0126 and 7ni on EGR-1 counts within the three nuclei that comprise the auditory thalamus combined. A one-way ANOVA produced a significant result ($F(2,11)=4.99, p<0.03$), where the animals that received intra-LA microinfusions of 7ni prior to conditioning showed attenuated EGR-1 expression when compared to vehicle controls (Dunnett's t, $p<0.02$). Thus, the results indicate that inhibition of LA-based nNOS activity but not LA based ERK signalling prevents conditioning induced EGR-1 upregulation in the auditory thalamus.

Discussion

In the previous experiment (Chapter 2) it was revealed that auditory fear conditioning significantly increased EGR-1 protein levels in the auditory thalamus. This series of experiments was conducted to determine whether the LA was regulating this activity via the retrograde signalling molecule NO. My results firstly revealed that pre-conditioning microinfusions of the $GABA_A$ receptor antagonist muscimol into the LA

significantly impaired training-induced EGR-1 upregulation. Secondly, the results showed that intra-LA microinfusions of the selective nNOS inhibitor 7-NI or the membrane impermeable NO scavenger cPTIO also impaired training-induced thalamic EGR-1 upregulation in a significant manner. Finally, an experiment was conducted to examine the time frame during which the LA is modulating EGR-1 expression in the auditory thalamus. The ERK inhibitor U0126 was microinfused into the LA prior to conditioning at a concentration previously shown to impair fear memory consolidation while leaving short term memory intact. The results showed that this manipulation had no effect on EGR-1 upregulation in auditory processing regions of the thalamus.

The above result that ERK inhibition in the LA did not significantly affect training-induced EGR-1 upregulation in the auditory thalamus is consistent with previous research where microinfusions of U0126 into the LA failed to impair training induced plasticity in the PP/PIN/MGM (Schafe, 2005). It has been reported that LA levels of phospo-ERK peak 60 minutes post conditioning (Schafe, 2000). In light of this, the results indicate that activation of LA intracellular pathways prior to this time is required for plasticity-induced changes in the auditory thalamus, or more specifically EGR-1 upregulation. This is inline with previous physiological research where functional inactivation of the amygdala during fear conditioning was reported to impair plasticity in the MGM/PIN during the acquisition phase of memory formation (Maren, 2001; Poremba, 2001). Furthermore, this is consistent with my finding that LA NO synthesis is required for training induced EGR-1 upregulation in the auditory thalamus. Neuronal-NOS is situated in close proximity to the NMDA receptor due to colocalization with PSD-95. Its activation appears to be temporally related to an increase in NMDA receptor channel conductance as it is indirectly activated by the calcium the passes through the activated receptor. Given the key role the NMDA

receptor plays in fear memory acquisition it stands to reason that NO signalling would also occur around that same time frame. Furthermore, the impairment induced by the membrane impermeable NO scavenger cPTIO suggests that NO's ability to modulate thalamic EGR-1 protein upregulation is dependent on its transition into the extracellular space, and thus suggests that it is acting in a presynaptic manner.

The finding that intra-LA microinfusions of muscimol impaired training induced EGR-1 upregulation in the auditory thalamus follow in the same vein as research by Poremba and colleagues (2001). They reported a significant impairment in discriminative training induced thalamic plasticity after pre-training intra-LA microinfusions of muscimol. In a similar manner, Huff and colleagues (2006) reported that $GABA_A$ activation in the LA impairs contextual fear conditioning induced increases in cFOS and Arc mRNA in the dorsal hippocampus. My results follow in the same vein as those mentioned above and illustrate that LA activation is necessary for conditioning induced upregulation of EGR-1 in the auditory thalamus. However, the results indicate that this is mediated by LA based NO synthesis, whereby NO acts as a retrograde messenger modulating intracellular activity in auditory thalamus.

Previous research has revealed that activation of the MAPK pathway in the auditory thalamus is required for fear memory consolidation. In fact, some NO targets are upstream of the MAPK cascade placing LA based NO in an excellent position to regulate this kinase pathway. In line with this, unpublished observations from our lab have revealed that 1) PKG inhibition in the LA impairs ERK phosphorylation in the auditory thalamus, and 2) inhibition of thalamic MAPK impairs training induced thalamic EGR-1 regulation. Collectively, these results point to the fact that LA-based NO is mediating EGR-1 upregulation via PKG and MAPK pathways.

However, there are other NO targets, or targets downstream of NO, that may be contributing to this increase in EGR-1 upregulation. For example, NO can directly interact with Ras a membrane bound GTPase directly upstream of the MAPK signalling pathway (Kushner, 2005; Lander, 1996, 1995; Yun, 1998). In fact, previous research has revealed that Ras activation in the amygdala is required for fear memory consolidation (Merino, 2006). Ras has been localized at synaptic terminals placing it in a valid position to mediate activation of presynaptic signalling cascades (Kushner, 2005; Mizoguchi, 1990; Mizoguchi, 1989; Mochly-Rosen, 1990). Given the fact that Ras is directly activated by NO via nitrosylation (Lander,1996, 1995; Yun, 1998), this may be a faster, or at least alternative, means by which the MAPK pathway is regulated in the auditory thalamus via NO signalling. Indeed, there is research that has shown that EGR-1 upregulation is downstream of Ras-dependent activation of the MAPK pathway (Alexandropoulos 1992; Cosgaya, 1999; Rusanescu, 1995). Further examination is required to determine whether Ras-induced modulation of EGR-1 occurs at thalamo-LA synapses and the temporal dynamics of this regulation when compared to the PKG pathway. These two NO based cascades may contribute to temporarily restricted IEG waves. Indeed, multiple waves of IEG transcription have been observed as a consequent of cellular stimulation (Clayton, 2000; Hemish, 2003). An alternative explanation is that these two pathways may work in unison to modulate the kinase cascade. Future research is required to examine these questions.

At the level of transcription, the NO-sGC-PKG pathway is known to contribute to CREB phosphorylation. Recently, it has been reported that an increase in CREB activity in the auditory thalamus enhances auditory fear memory formation (Han, 2008). The EGR-1 promoter contains a CRE binding element indicating that EGR-1 upregulation is under the control of CREB. Future investigations are required to

determine whether this is indeed the case within the thalamo-LA circuitry. Further, it seems plausible that an increase in CREB activity in the auditory thalamus would be correlated with an increase in training induced EGR-1 upregulation.

Beyond modulation of intracellular activity NO is also capable of regulating the extracellular concentration of various neurotransmitters including dopamine (DA) and NE. An increase in the extracellular concentration of NE has been attributed to a LA based posttranslational modulation of mRNA in the hippocampus. Given NO's ability to modulate the extracellular concentration of NE, it is plausible that this is a further facet of NO-based signalling modulating gene expression. In line with this, there is research that suggests that the NE and DA system plays a role in EGR-1 upregulation (Bhat, 1993, 1992; Shirayama, 2000). However, the role of the NE system is controversial (Bhat, 1993). Nonetheless, the important point here is that the role of NO signalling is by no means straight forward. As discussed above, there are numerous mechanisms by which NO may contribute to thalamic EGR-1 upregulation beyond that of the typical PKG pathway investigated by many. However, the important point that this research illustrates is that the LA is modulating afferent brain areas during fear memory acquisition via NO signalling, presumably in a temporally restricted manner to NMDA receptor activation.

B. NR2B-NMDA receptor activation in the Amygdala is required for training induced thalamic EGR-1 upregulation

Electrophysiological and behavioural pharmacological studies have shown that intra-LA activation of the NMDA receptor is required for fear conditioning (Bauer, 2002; Blair, 2001;Campeau, 1992; Fanselow, 1994; Lee, 1990). In order for NMDA receptor activation two requirements above and beyond routine cellular basal activity must be met; firstly glutamate must be bound to the receptor, secondly the membrane in which the receptor is situated must be sufficiently depolarized. Together these events induce a structural change in the receptor and expulsion of a magnesium ion from the receptor channel leading to receptor activation. Because of these two requirements for activation, these ionotropic receptors are characteristically referred to as coincidence detectors because both the pre and postsynaptic cell must be simultaneously activated for NMDA channel opening (Blair, 2001).

The typical auditory fear conditioning procedures involves presentation of a tone-CS for 20 seconds, which coterminated with a 1 second footshock-US. Together these two stimuli are thought to satisfy the requirement for NMDA receptor activation. The presentation of the CS induces glutamate release from auditory thalamic terminals, which binds to both AMPA and NMDA glutamatergic receptors. At this stage the CS-induced excitatory post synaptic potential is primarily caused by AMPA receptor activation and thought to be small and brief. The NMDA receptors fail to activate as the membrane depolarization is not substantial enough to liberate the magnesium ion from the receptor channel. Even though the receptors fail to activate, one of the prerequisites for their activation is met; glutamate is bound to the receptor. Subsequent presentation of the US results in excitatory neurotransmission that strongly depolarizes some of the

same cells in the LA that were depolarized by the CS (Romanski, 1993). As a result, within these cells the NMDA receptor is unblocked and consequently activated (Blair, 2001). NMDA receptor activation increases the channels permeability to excitatory ions including calcium, which move down their concentration gradient into the cell. As mentioned in the introduction, an increase in intracellular calcium concentration is pivotal for initiation of intracellular events critical for synaptic plasticity or more specifically memory formation and subsequent consolidation. However, given the permeability of the NMDA receptor to positive ions including calcium, it is possible that the receptor could contribute to cellular excitation and thus fear related neurotransmission.

In an effort to establish the role played by LA-based NMDA receptors in fear conditioning researchers have compared the deficit induced by NMDA receptor antagonism prior to conditioning to deficits induced by antagonism prior to fear memory recall. It is thought that behavioural impairments that result from preconditioning blockade can only be attributed to memory processes if the same manipulation prior to fear expression fails to produce an effect. Experiments where pre-conditioning NMDA receptor antagonism was employed have unequivocally shown, as mentioned above, that NMDA receptor activation is required for fear memory acquisition and, by extension, consolidation (Bauer, 2002; Pistell, 2008; Walker, 2000). However, the role of the receptor in fear memory expression is less clear cut.

A typical NMDA receptor functions as a heteromeric complex comprised of two glycine binding N1 subunits and least one glutamate binding N2 subunits (Laube, 1998). The type of N2 subunit (NR2A-NR2D) determines the biophysical and pharmacological properties of the different NMDA receptors, including opening probability, deactivation time, single channel conductance, and interactions with

intracellular signalling molecules (Cull-Candy, 2004). For example, NMDA receptors containing a NR2B subunit have slower deactivation kinetics and carry more calcium charge per unit current than those that contain a NR2A subunit (Chen, 1999; Cull-Candie, 2001; Monyer, 1994; Sobczyk, 2005). At thalamo-LA synapses NMDA receptors with NR2A and NR2B subunits dominate (Weisskopf, 1999). Recently, researchers have used selective NMDA receptor antagonists to investigate the role of these distinct NMDA receptor types in fear memory processes.

Behavioural experiments have suggested that the NR2B receptor types underpin fear memory consolidation while NR2A isoforms play a more predominant role in fear related neurotransmission. For example, pre conditioning intra-LA microinfusions of an NR2B receptor antagonist prevents fear memory consolidation to either a tone or light, while having no affect on conditioned fear expression (Rodrigues, 2001; Walker, 2008). Selective blockade of NR2A subtypes, on the other hand, does interfere with conditioned fear memory expression, illustrating their role in fear related neurotransmission (Walker, 2008). Interestingly, the involvement of NR2B subtypes in fear memory consolidation is dependent on the strength of the conditioning protocol. Selective intra- LA blockade, or genetic knockdown, of NR2B subtypes impairs auditory conditioned fear when five tone-shock pairings are presented, but fails to induce an effect when a weaker one tone-shock pairing is employed. In accordance with their more ubiquitous role in fear related neurotransmission, antagonism of NR2A receptor types impaired fear memory consolidation during both conditioning protocols (Zhang, 2008). Given the NR2B receptors lower opening probability in comparison to the NR2A (Erreger, 2005), the authors propose that the glutamate released during one tone-shock paring is not sufficient to recruit NR2B receptor types (Zhang, 2008). Nonetheless, the more typical conditioning paradigm, as primarily employed in the

research, involves three or more tone shock pairing and thus is predominantly reliant on NR2B-NMDA receptor activation.

Cell signalling downstream of NMDA receptor activation

All NMDA receptor types are localized within a dense network of macromolecular signalling molecules at the post-synaptic-density (Ziff, 1997). The close association of the receptor with second messenger signalling proteins serves to facilitate NMDA receptor-mediated second messenger synaptic signalling. Neuronal-NOS colocalizes with both NR2B- and NR2A- NMDA receptor types via the scaffold protein PSD-95 (Al-Hallaq, 2007; Christopherson, 1999; Garner, 2000; Kornau, 1995). Therefore, NO synthesis as a result of NMDA receptor activation may act as an intermediatory between receptor activation and cellular intracellular signalling cascades required for fear memory consolation including presynaptic EGR-1 upregulation.

Numerous studies have shown that EGR-1 upregulation is downstream of NMDA receptor activation (Beckmann, 1997; Cole, 1989; Condorelli, 1994; Szekely, 1990; Williams, 2000). For example, 3,4-methylenedioxymethamphetamine (MDMA)-induced EGR-1 mRNA upregulation (which occurs in the prefrontal cortex, striatum, and hippocampal dentate gyrus) is significantly attenuated by an NMDA receptor antagonist (Shirayama, 2000). In the fear system, intra-LA EGR-1 mRNA upregulation is dependent on NMDA receptor activation (Malkani, 2001). However, presynaptic EGR-1 upregulation relative to postsynaptic NMDA receptor activation has not been reported. There is evidence that this signalling pathway could exist and LA-thalamo synapses. For example, there are cell signalling components' in the auditory thalamus known to play a role in fear conditioning that lie in between NMDA receptor activation and EGR-1 protein upregulation. The research results above (Chapter 3) have shown

that EGR-1 protein upregulation is downstream nNOS activation. Other researchers' have shown that its upregulation is downstream of MAPK and CREB (Cruzalegui, 1999; Davis, 2000; Greenwood, 2002; Jannsen-Timmen, 1989; Sgambato, 1998; Whitmarsh, 1995), again, both of which lie downstream of NMDA receptor activation. Thus, it seems possible that conditioning induced EGR-1 upregulation in the auditory thalamus may be downstream of NMDA receptor activation. Moreover, given the role of retrograde signalling at LA-thalamo synapses it is quite likely that the NMDA receptors are LA based.

This current study

The following experiment was conducted to assess whether activation of intra-LA NR2B-NMDA receptors is required for conditioned induced EGR-1 upregulation in the auditory thalamus. Selective inhibition of NR2B-NMDA receptors was conducted as they play a more intimate role in memory consolidation and are not required for fear related neurotransmission at LA-thalamo synapses (Bauer, 2002). As with the previous studies, a three tone-shock pairing protocol thus ensuring that fear memory formation was NR2B-NMDA receptor dependent. To this end, the selective NR2B receptor agonist, ifenprodil (Legendre, 1991, Reynolds, 1989), was microinfused into the LA prior to auditory fear conditioning and examination of intra-thalamic EGR-1 protein levels across the PP, PIN, and MGM two hours later was conducted. If activation of NMDA-NR2B receptors are indeed upstream of EGR-1 upregulation in the auditory thalamus it is anticipated that a significant degree of EGR-1 downregulation in animals who received intra-LA microinfusions compared to vehicle-controls.

Method

Subjects

Ten adult (300-350g) male Sprague Dawley Rats (Hilltop Laboratories, Philadelphia, PA) served as subjects. They were housed individual in plastic cages with free access to food and water. The colony was maintained on a 12 hr light dark cycle.

Surgery

Rats were anethnatised with a ketamine (95mg/Kg) and xylazine (0.05mg/kg) mixed solution, mounted in a sterotaxic and implanted with guide cannulas (26 gauge; Plastics One, Roanoke, VA) aimed at the LA (AP: -3.3, ML: ±5.0, DV: -8.0). The implants were secured to the skull with acrylic cement and jeweller screws. Immediately after surgery animals received an IP administration of buprenex (0.5ml 0.03mg/ml). Three days following surgery ibuprofen was freely available to the animals in their water supply.

Drugs.

Ifenprodil (1.0µg/0.5µl) was dissolved in a 2% 2-hydroxy-β-cylodextran (HBC; Sigma Aldrich) saline vehicle.

Behavioural Procedures

On two consecutive days prior to training the animals were habituated to handling and dummy cannulae removal. On training day animals were handheld and the dummy cannulae removed. Animals were randomly assigned to the Ifenprodil (n=5) or Vehicle control (n=5) condition. Infusion cannulas with 1mm projections below the tip of the guide were inserted and either ifenprodil or vehicle only were infused

(0.2µl/min), once infusions was complete the infusion cannulae were retained in the guide for a further 1min to allow drug diffusion from the tip.

Behavioural training commenced thirty minutes after drug infusion. Rats were placed in the conditioning chambers and allowed 150 sec to acclimate. They were then presented with three tone-shock pairings at an average ISI of 120 sec. The tone (5kHz, 75 dB) was presented for 20 seconds and coterminated with a 1 sec 1 mA footshock. Total training time was 9 mins.

Two hours after training rats were euthanized with chlorohydrate (250 mg/kg, IP administration). Then transcardially perfused with ~50ml of PBS followed by 250ml of ice cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The brains were promptly removed and postfixed in 4% paraformaldehyde-PB solution overnight then transferred into 20% glycerol-0.1 M PB cryoprotectant solution and stored at 4ºC until sectioning (see Figure 5, section A of this chapter for a schematic of the training protocol). .

Immunohistochemistry

Methods were exactly the same as outline in section A of this chapter above.

Data analysis

Thalamic sections between -5.3 and -6.04 relative to bregma were selected for scoring. Cell counts were taken from approximately four sections per animal, and scored using a defined boundary around the PP, PIN, and MGM using ImagePro (Media Cybernetics, Silver Spring, MD). For analysis, for each animal left and right hemispheres' were averaged, then an average for an animal based on all sections was determined, give a single score for each animal. Group means were then calculated. Results were analysed using students *t* instead of Dunnett's as employed in the previous

results analysis as Dunnett's can only be used when there is one reference group compared to more than one control group. In this experiment two groups were used.

During training movement amplitudes prior to the 1 sec shock presentation and 20 sec post shock presentation were recorded. Total seconds of freezing during each time period were recorded and expressed as a percentage of behavioural freezing. Results were compared using *t*-tests.

Results

Histology

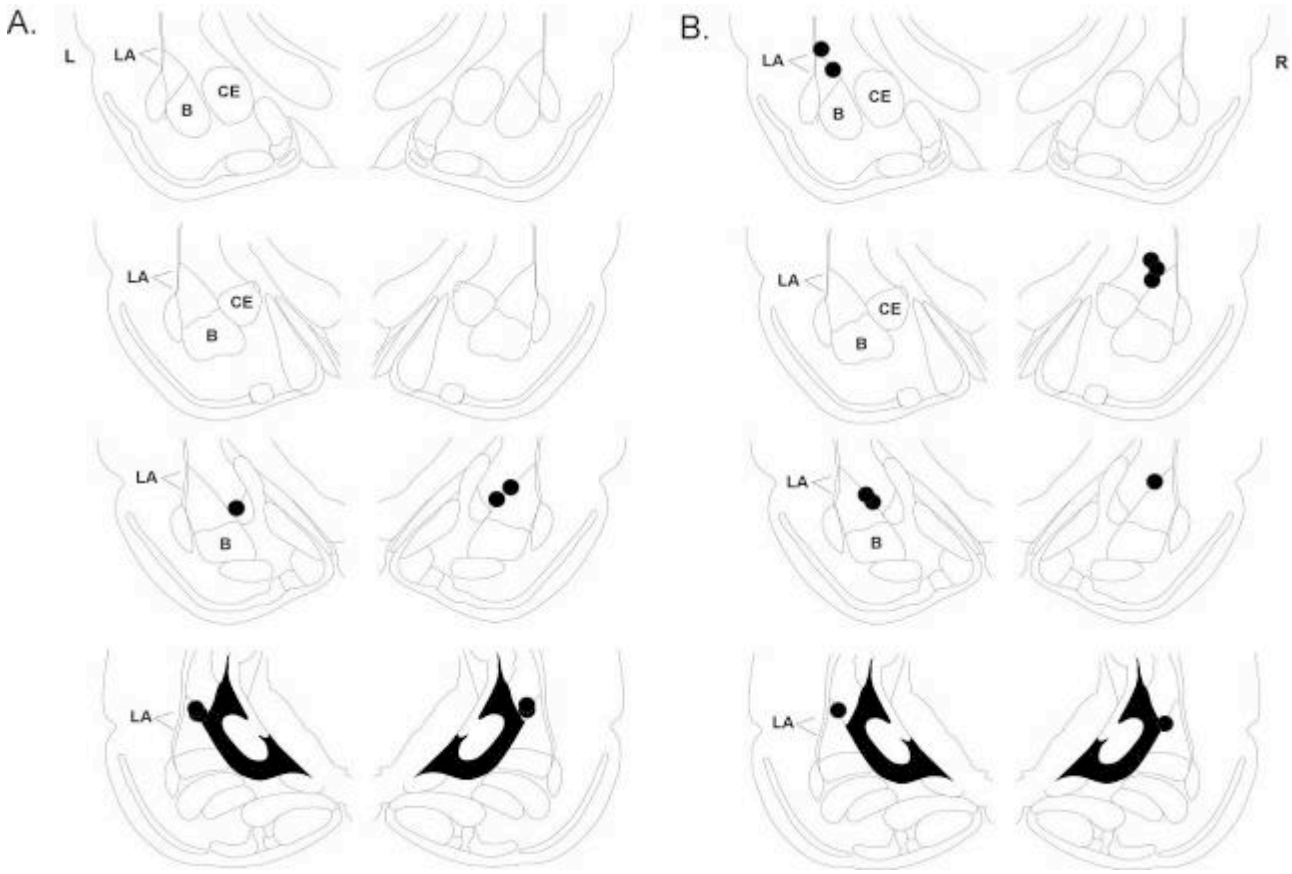

Figure 14. Schematic coronal sections through the rat brain showing the intra-LA cannula tips in animals who received (A) Ifenprodil (n=6) or (B) vehicle (n=4) microinfusions. The arrows depict the location of the LA. Schematics are adapted from the rat brain atlas (Paxinos, 2004). LA: lateral amygdala, B: Basal amygdala, CE: central nucleus of the amygdala.

Figure 14 shows the location of intra-LA infusion cannula tips for animals that received intra-LA ifenprodil or vehicle microinfusions. All placements are superimposed on coronal sections taken from the rat brain atlas (Paxinos, 2004). As illustrated, the cannula placements were predominantly in the medial lateral portion of the LA.

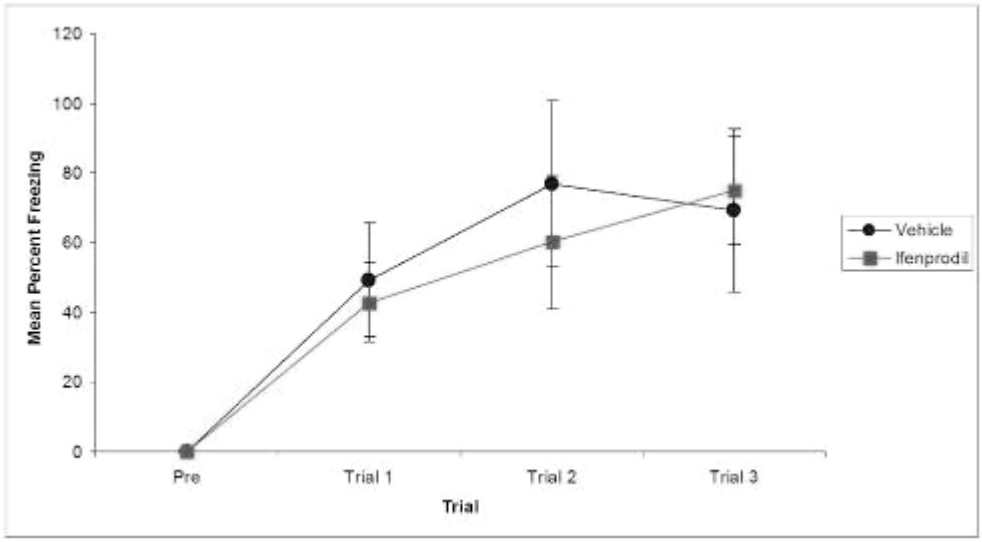

Figure 15. Average postshock freezing (± SEM) measured prior to shock presentation (Pre) and for each of the 3 conditioning trails 20 sec immediately following shock termination as a function of intra-LA microinfusions of ifenprodil or vehicle control.

Figure 15 depicts the average percent freezing during shock presentation and across each of the three conditioning trials for animals that received intra-LA microinfusions of ifenprodil or vehicle. Both groups appeared to have equivalent and intact levels of postshock freezing. Independent sample *t*-tests comparing the level of freezing between each group for each trial revealed no significant differences (Trial 1 p=0.3; Trial 2 p=0.7; Trial 3 p=0.8). Overall, both groups exhibited intact postshock freezing and thus normal footshock perception.

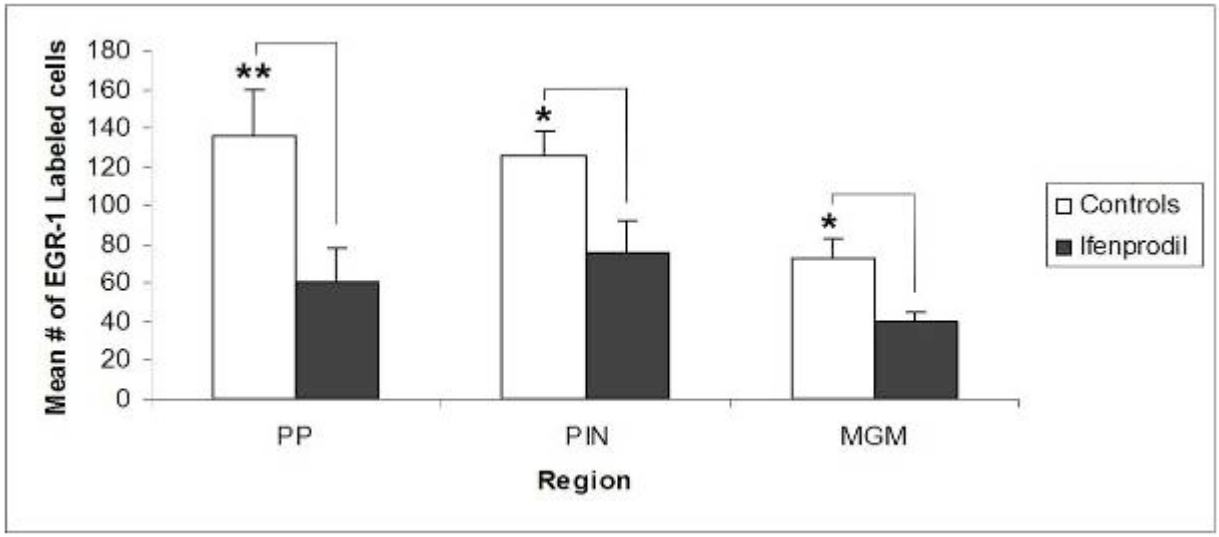

Figure 16. Mean EGR-1 count across the nuclei of the auditory thalamus; PP, PIN,MGM in animals who received intra LA microinfusions of Ifenprodil or Vehicle prior to receiving three tone-shock pairings. *p<0.05, **p<0.001.

The results in Figure 16 reveal that intra-LA microinfusions of Ifenprodil reduced training induced EGR-1 protein levels across all nuclei of the auditory thalamus. Analysis of the differences using independent sample *t*-tests confirmed that the differences across the PP ($t(8)=8.22, p< 0.001$), PIN ($t(8)=2.67, p<0.03$), and MGM ($t(8)=2.72, p<0.03$) were all significant.

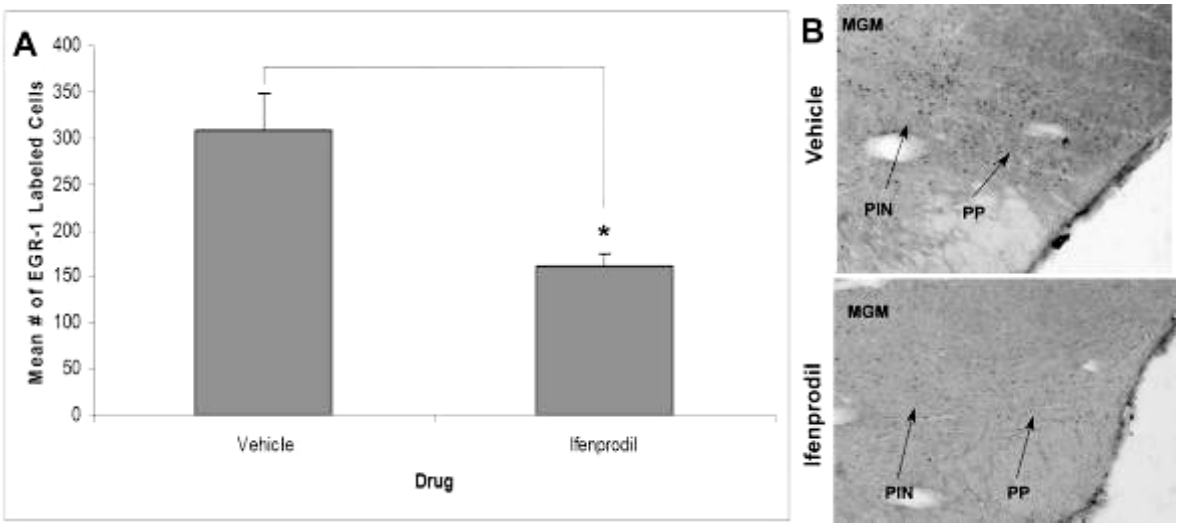

Figure 17. **A.** Mean EGR-1 cell count (+SEM) across the PP, PIN, and MGM combined in vehicle controls or animals who received ifenprodil (1.0µg/0.5µl) prior to 3 tone shock conditioning trials. * $p<0.05$ **B.** Representative micrographs of immunoreactivity in the auditory thalamus for the two conditions.

For further analysis EGR-1 counts across all nuclei that comprise the auditory thalamus were combined. The results are depicted in Figure 17. Again, results were analysed using an independent *t*-test. The difference between the Ifenprodil and Vehicle group proved to be significant ($t(8)=3.55, p<0.008$).

Discussion

The aim of this experiment was to determine whether intra LA blockade of NMDA-NR2B receptors affected conditioning induced EGR-1 upregulation in auditory thalamic cells. NR2B-NMDA receptor activation was inhibited as previous research has shown that 1) they are predominantly localized with thalamo-LA synapses (Radley, 2007) and 2) that their antagonism impairs fear memory consolidation while leaving fear expression intact, thus suggesting that they play a more predominant role in fear memory associated processes than processes associated with neurotransmission (Rodrigues, 2001). The results indicate that intra-LA microinfusions of the selective NMDA-NR2B receptor antagonist ifenprodil, significantly reduced training induced

EGR-1 upregulation across the PP, PIN, and MGM of the thalamus. This revealed that LA based NR2B-NMDA receptor activation is upstream of training induced increases of EGR-1 in the auditory thalamus. Given my previous findings it is likely that NO works as a retrograde signalling molecule at LA-thalamo synapses acting as in intermediatory between NR2B-NMDA receptor activation and gene upregulation in the auditory thalamus.

An interesting question to ask is whether antagonism of NR2A-NMDA receptors in the LA prior to fear conditioning would also impair EGR-1 protein upregulation in the auditory thalamus? Indeed, NR2A-NMDA receptors have also been localized within the LA. Further, nNOS is known to colocalize with the NR2A splice variant. In a recent study, Al-Hallaq and colleagues (2007) have reported that NR2A receptor type colocalizes with nNOS to a greater degree than NR2B in hippocampal tissue (Al-Hallaq, 2007). In order to come to this conclusion the authors eliminated tri-heteromeric complexes (NR1/NR2A/NR2B) from their analyses and thus focused their examination of di-heteromeric (NR1/NR2A and NR1/NR2B) receptor populations. The fact that tri-heteromeric complexes exist (Luo, 1997; Sheng, 1994), raises a further couple of important questions; firstly, do tri-heteromeric complexes exist in the LA, and if so what is their density. Secondly, and most importantly for my research findings, does ifenprodil antagonize tri-heteromeric complexes? It has been reported that ifenprodil selectively binds to NR2B subunits of NMDA receptors, antagonizing the receptor (Legendre, 1991; Reynolds, 1989). However, NR2B-NMDA receptors form a component of tri-heteromeric NMDA receptor complexes. It would be interesting, and pertinent in delineating research findings concerning ifenprodil, to investigate whether its selective antagonisms of the NR2B unit is equivalent in both tri-heteromeric and di-heteromeric NMDA receptor populations. However, these points

should not detract from the fact that the above results did, indeed, show a significant regulatory affect of EGR-1 protein expression induced by NR2B-NMDA receptor antagonism. Thus, revealing that LA localized NMDA receptor activation mediates presynaptic modulation of intracellular signalling cascades during fear memory consolidation.

Intra-LA NMDA receptor activation is known to play a role in a number of different fear memory processes beyond initial consolidation, including extinction, memory reconsolidation, and higher order processes such as second order fear conditioning (Ben Mamou, 2006). Future investigations are required of whether NMDA-NO signalling plays a role in modulating intra cellular signalling and gene expression associated with these processes. Furthermore, investigations into whether this occurs in a presynaptic manner would aid in the understanding of the breadth of LA modulation of afferent neuroanatomical structures during conditioned fear associated processes.

The results of this experiment have indicated that NMDA-NR2B receptor activation is required for training induced EGR-1 upregulation in the auditory thalamus. Collectively, the results of this chapter suggest that the LA is modulating EGR-1 upregulation. This is occurring downstream of NR2B-NMDA receptor activation during the acquisition phase of fear memory formation. This requires nNOS activation and signalling via NO within the extracellular space, which accordingly suggests that NO is acting as a retrograde signalling molecule at thalamo-LA synapses. However, it must be noted that LA modulation of training induced thalamic EGR-1 upregulation could be occurring via a transynaptic mechanism. For example, it is possible that LA based NO synthesis could be modulating cortical neuronal inputs to the LA. This in turn could eventuate with cortical based modulation of thalamic EGR-1. In the next

chapter research was conducted were cells in the auditory thalamus that specifically project to the LA were isolated, training induced EGR-1 upregulation in this specific population was then examined.

Chapter 4

Thalamic cells that project to the LA are EGR-1 positive

There is a plethora of research showing that cells of the auditory thalamus project to the LA (Bordi, 1994; Doron, 1999; Farb, 1997; LeDoux, 1991). With regard to fear conditioning one of the primary functions of this area is to relay auditory associated neurotransmission to the amygdala (Campeau, 1995; Jarrell, 1986; LeDoux, 1986; LeDoux, 1986). However, the results of this dissertation have demonstrated that signalling at thalamo-LA synapses can occur in a retrograde type manner. That is, pharmacological manipulation of the LA can modulate the expression of EGR-1 in the auditory thalamus. However, there is no clear anatomical evidence indicating neuroanatomical connectivity between auditory thalamic cells that express training induced EGR-1 and those that form presynaptic connections within the LA.

The results of Chapter 2 demonstrated that a paired protocol, while producing a greater degree of EGR-1 upregulation in the auditory thalamus, failed to produce a level of EGR-1 upregulation that differed significantly from an Immediate-shock or Unpaired group. The important point here is that presentation of stimuli in an immediate shock protocol does not result in the formation of a memory association (Fanselow, 1980). While such IEG upregulation could be indicative of a memory priming mechanism, which is, however, not sufficient for formation of a fear association, it has been suggested that when an animal is presented with such stimuli it does not mean that the animal do not attempt to form an association not directly measured by the experimenter (Clayton, 2000). That is, EGR-1 upregulation in the auditory thalamus could subserve memory associations' beyond fear conditioning. Clayton (2000) states, "in theory if one could isolate the hypothetical circuit engram

that would uniquely specify a particular learned association one might be able to probe the interactions between the initiating synaptic activity the presence of absence of a rise in IEG expression and a subsequent change in synaptic activity in the engram." Thus, analysis of cells within the auditory thalamus that specifically project to the LA may produce slightly different or more specifically, significant differences between animals presented with paired stimuli presentation and control groups. To this end, in the present experiments' auditory thalamic cells that project to the LA were localized and specifically analysed for training induced EGR-1 upregulation.

Experimental procedures employed in this current study

In the current study the fluorescent retrograde signalling molecule fluorogold (FG) was used. However, FG was not localized in the auditory thalamus using its fluorescent properties but instead identified using immunohistochemistry (IHC). The advantages of identifying FG positive cells in this manner are 1) an increase in sensitivity and 2) tissue staining that lasts indefinitely. In addition, because a double labelling experiment was conducted where both EGR-1 positive and FG positive cells were being analyzed adoption of such a method allowed selection of two coloured substrates that would allow for a greater degree of contrast between the two antigens of interest. An alkaline phosphate and phosphatase systems with blue and red substrates respectively was employed. A blue alkaline phosphatase substrate was used to detect the presence of EGR-1 protein within the cells, while a red peroxidase substrate was used to detect the FG label. An advantage of using alkaline phosphatase and peroxidase substrates together is that it eliminates the possibility of cross reactivity between antigens.

This current study

Our previous findings have shown that training induces a significant increase in EGR-1 protein in the auditory thalamus. This upregulation is dependent on LA based NMDA receptor activation and NO synthesis during the acquisition phase of fear memory formation. This series of experiments examined whether conditioned induced EGR-1 upregulation in the auditory thalamus is indeed occurring in cells that project to the LA. To this end, the retrograde tracer FG was microinfused into the LA 10-14 days prior to conditioning, animals were then trained with either three tone-shock pairings, a Paired group; subjected to three immediate shocks, Immediate shock group; or simply left in their home cages, acting as Naïve-controls. The number of EGR-1 positive cells in the PP/PIN/MGm were then quantified, followed by an analysis of the number of FG projection cells in the auditory thalamus. Finally, a count of the number of FG projection cells in the auditory thalamus that were also EGR-1 positive was performed. It is hypothesised that the LA is indeed regulating EGR-1 expression during training because of such it is expected that a significantly larger number of double labelled thalamic LA projection cells in Paired animals compared to the Immediate Shock and Naïve controls will be observed.

Method

Subjects

Seventeen adult (300-350g) male Sprague Dawley rats (Hilltop Laboratories, Philadelphia, PA) served as subjects. They were housed individually in plexiglass cages in a vivarium maintained on a 12 h light/dark cycle. All procedures were conducted during the light phase of the cycle. Food was available *ad libitum* throughout the experiment.

Surgery

Rats were anethnatised with a ketamine (95mg/Kg) and xylazine (0.05mg/kg) mix solution, mounted in a sterotaxic and implanted with guide cannulas (26 gauge; Plastics One, Roanoke, VA) aimed at the LA (AP: -3.3, ML: ±5.0, DV: -8.0). The implants were secured to the skull with acrylic cement and jeweller screws. Immediately post surgery animals received an IP administration of buprenex (0.5ml 0.03mg/ml). Three days following surgery, ibuprofen was freely available to the animals in their water supply.

Drugs.

FG (Fluorochrome, LLC, Denver Colorado) was dissolved in physiological 9% saline to produce a 2% solution.

Behavioural procedures

Approximately 4 days post surgery, animals were handheld and the dummy cannulae removed, infusion cannulas with 1mm projections below the tip of the guide were inserted and rats received intra-LA pressure infusions of the retrograde tracer FG (2%; 0.3 µl/side). Once infusion was complete the infusion cannulae were retained in the guide for a further 2 min to allow diffusion from the tip.

10-14 days post FG infusion the animals were habituated to handling, the following day rats were conditioned (*n*=7) or received 3 immediate shocks (*n*=6), the remaining animals (*n*=4) served as naïve controls. The experimental protocols were identical to those used in the previous experiments. In brief, the conditioning trails were three 20 sec tone (5kHz, 75 dB) presentations, which coterminated with a 1sec 1mA foot shock. The stimuli were presented at an average ISI of 120 min after a 150sec acclimation period. Animals in the Immediate Shock group were placed in the

conditioning chamber and received 3, 1mA, footshocks at an ISI of 0.5 sec. Most importantly, there was no acclimation period. After shock presentation they remained in the conditioning chambers an equivalent amount of time to those who were presented with a paired protocol. Thus, the training time across both conditions was 9 min.

Two hours following training, animals were rapidly and deeply anesthetized with chloral hydrate (250 mg/kg, I.P) and transcardially perfused with ~50ml of ice-cold PBS, followed by 250ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were then removed and post-fixed in 4% paraformaldehyde-PB for 24 hr and then cryoprotected in a 20% glycerol-0.1 M PB for 48 hr. See Figure 18 for a schematic of the behavioural procedures used in this experiment.

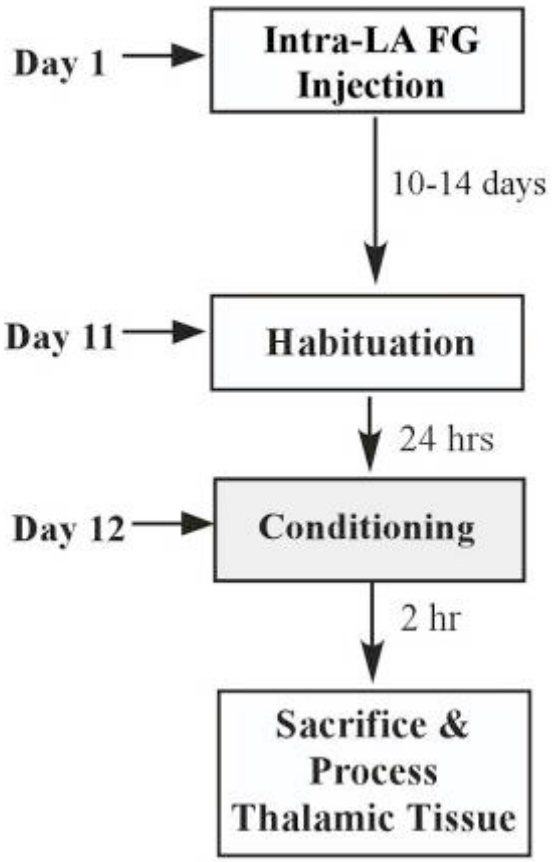

Figure 18. Schematic of the behavioural procedure sued to examine EGR-1 upregulation in LA projection cells. Animals received intra-LA microinfusions of the FG 10-14 days prior to training. The day before training the animals were habituated to handling. The animals were conditioned and then sacrificed for FG and EGR-1 immunoreactivity in the auditory thalamus.

Free-floating sections, 20 µm, through the posterior thalamus (PP/PIN/MGM); or 40 µm through the LA were cut using a sliding microtome and stored in a 1% NaAz-PBS solution. Approximately, 7 random thalamic sections from each animal were assayed for both EGR-1 and FG immunoreactivity.

Immunohistochemistry

The EGR-1 assay was completed first. The assay was completed in the same manner as described in the previous experiments. Except, sections were visualized using VectorStain ABC-Alkaline phosphatase kit (Rabbit IgG, Vector Laboratories, Burlingame, CA) and developed in darkness for 30 min using VECTOR Blue substrate.

For subsequent FG localization sections were blocked for an hour in PBS containing 1% bovine serum albumin (BSA)-0.1% Triton X-100, slices were incubated at room temperature overnight in anti-Fluorogold antibody (1:50,000; Flourochrome, LLC, Denver Colorodo) in PBS-1% BSA-0.1% Triton X-100. After extensive washes in PBS, sections were incubated for an hour in a biotin-conjugated goat anit-rabbit IgG2 and then visualized using VectorStain ABC kit (Vector Laboratories, Burlingame, CA) and developed in Vector NovaRED peroxidase substrate for 1 min. Sections were washed and mounted on Fisherbrand electrostatic slides using an aqueous mounting solution and cover slipped.

For localization of Fluorogold diffusion placement within the LA, sections were processed for FG IHC analysis as described above then mounted and cover slipped for later viewing.

Data analysis

Thalamic sections between -5.3 and -6.04 relative to bregma were selected for scoring. Cell counts were taken from at least four sections per rat and scored using a defined boundary around the PP, PIN, or MGM using light microscopy. The analysis involved a count of the total number of EGR-1 positive cells in each structure, followed by the number of FG positive cells. Finally, the number of double labelled cells expressing both EGR-1 and FG stain were determined and then expressed as a percentage of the total number of FG positive cells. For each animal, cells were quantified within both the left and right hemispheres and then average to give a single score, group averages were then determined and analyzed using ANOVA and *post hoc* *t*-tests.

LA sections were examined for FG diffusion within the LA. Animals with FG injection sites not localized to the LA were removed from the analysis

Results

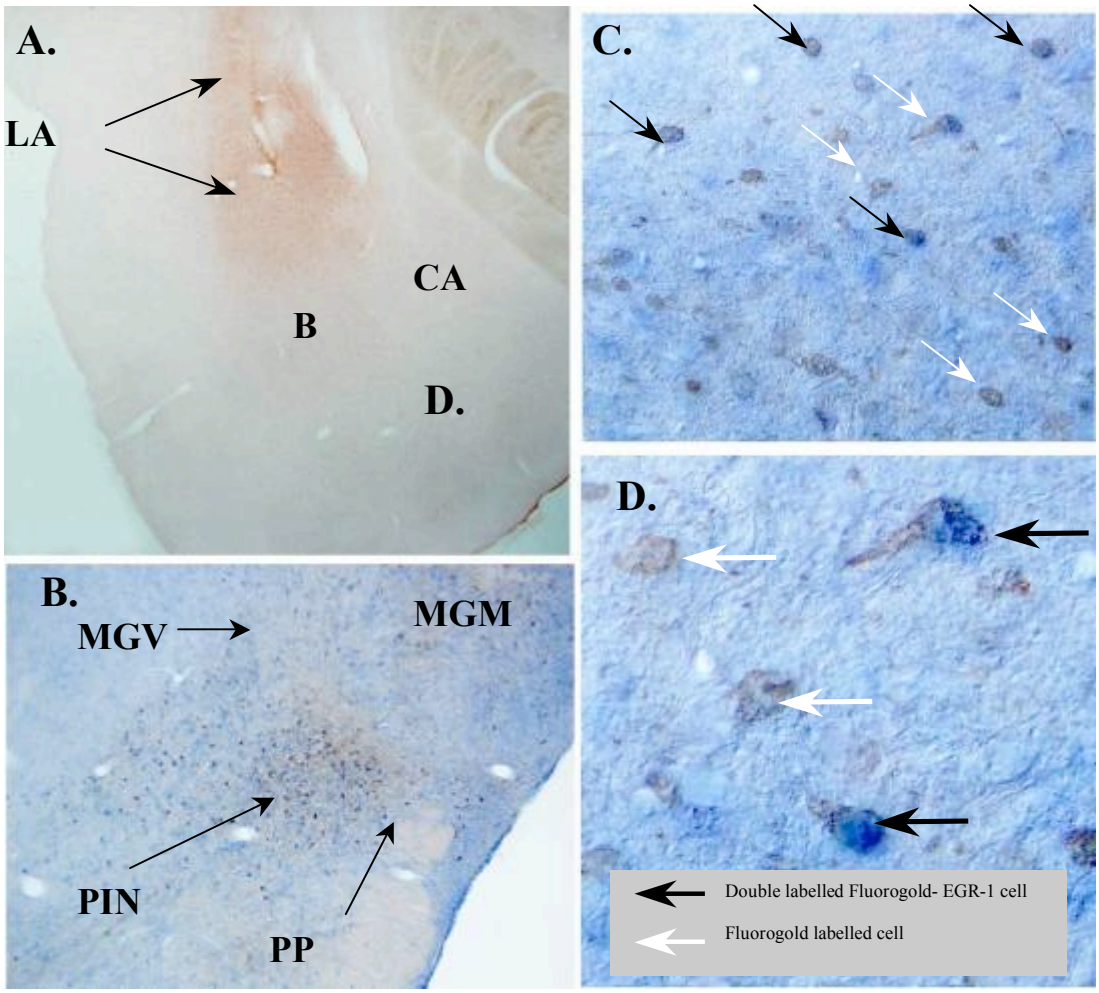

Figure 19. A representative example of IHC analysis of FG diffusion within the LA and FG and EGR-1 immunoreactivity in the auditory thalamus. (A) Immunoreactivity for FG in the LA 10-14 days post infusion. (B) Micrograph of the auditory thalamus illustrating dense double label within the PP and PIN. (C, D) successively higher magnifications of the PIN region showing double label and FG alone label. (LA: Lateral nucleus of the amygdala, CA: Central nucleus of the amygdala, B: Basal nucleus of the amygdala, MGM: Medial geniculate medial, MGv: Medial geniculate ventral, posterior intralaminar nucleus, PP: peripeduncular nucleus).

Figure 19 shows representative micrographs of immunoreactivity for FG within the LA. It also shows FG (red stain) and EGR-1 (blue nuclear stain) in the auditory thalamus. Throughout all sections the FG positive cells were densely localized within the PP and PIN, as were EGR-1 labelled cells.

Figure 20 a-c show representative examples of schematics of both diffusion and label in the LA and auditory thalamus respectively in a Paired, Immediate Shock, and Naïve animal. As depicted FG diffusions were predominantly concentrated within the

LA importantly there were no systematic differences in diffusion dynamics between the three groups.

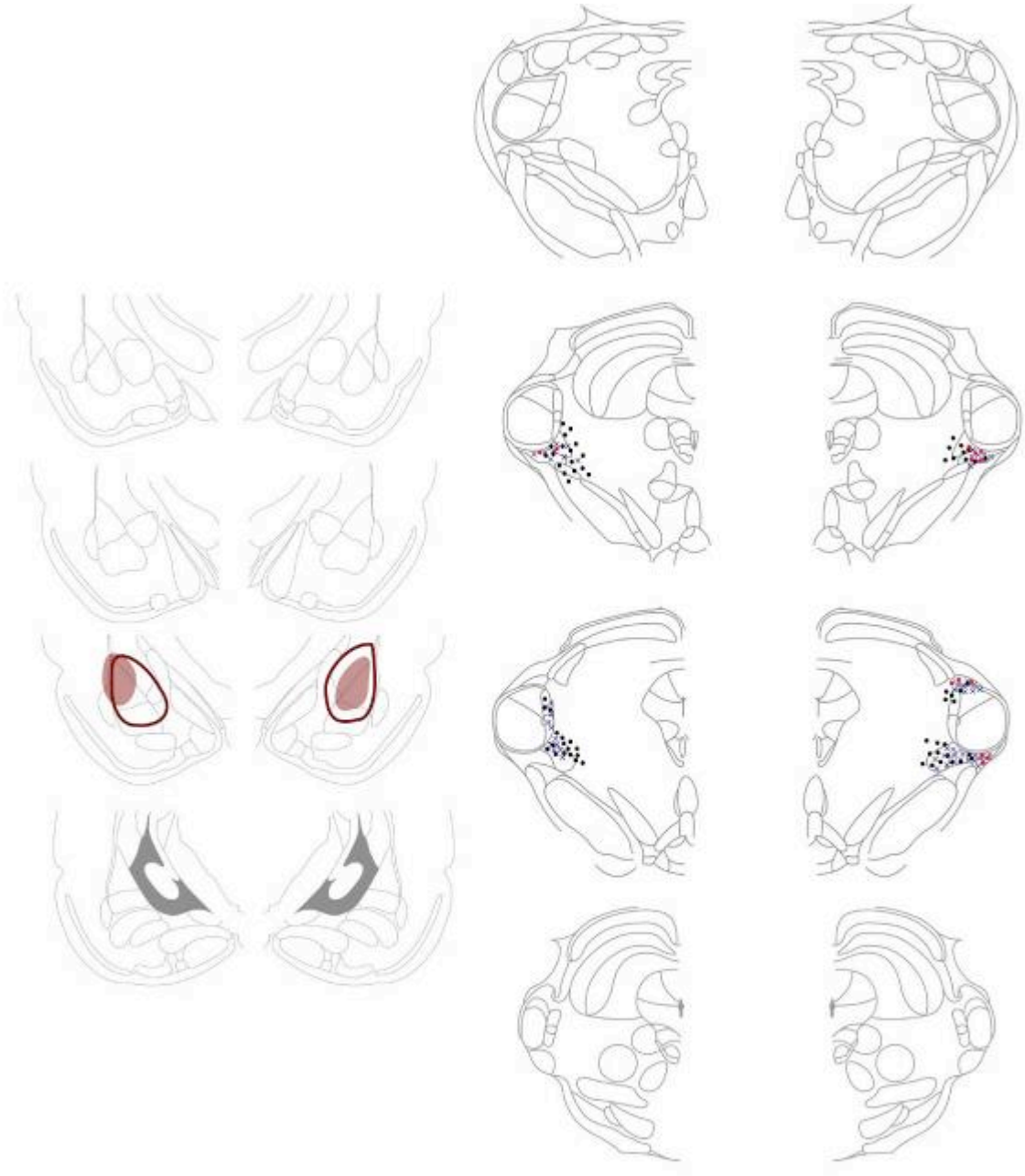

Figure 20a Representative example of a schematic for a Paired animal. Figure A shows the largest and smallest diffusion of FG in the LA and B) the corresponding label in the auditory thalamus. (Blue cross = FG label, Black dot = EGR-1 label, Red dot = Double labelled cell).

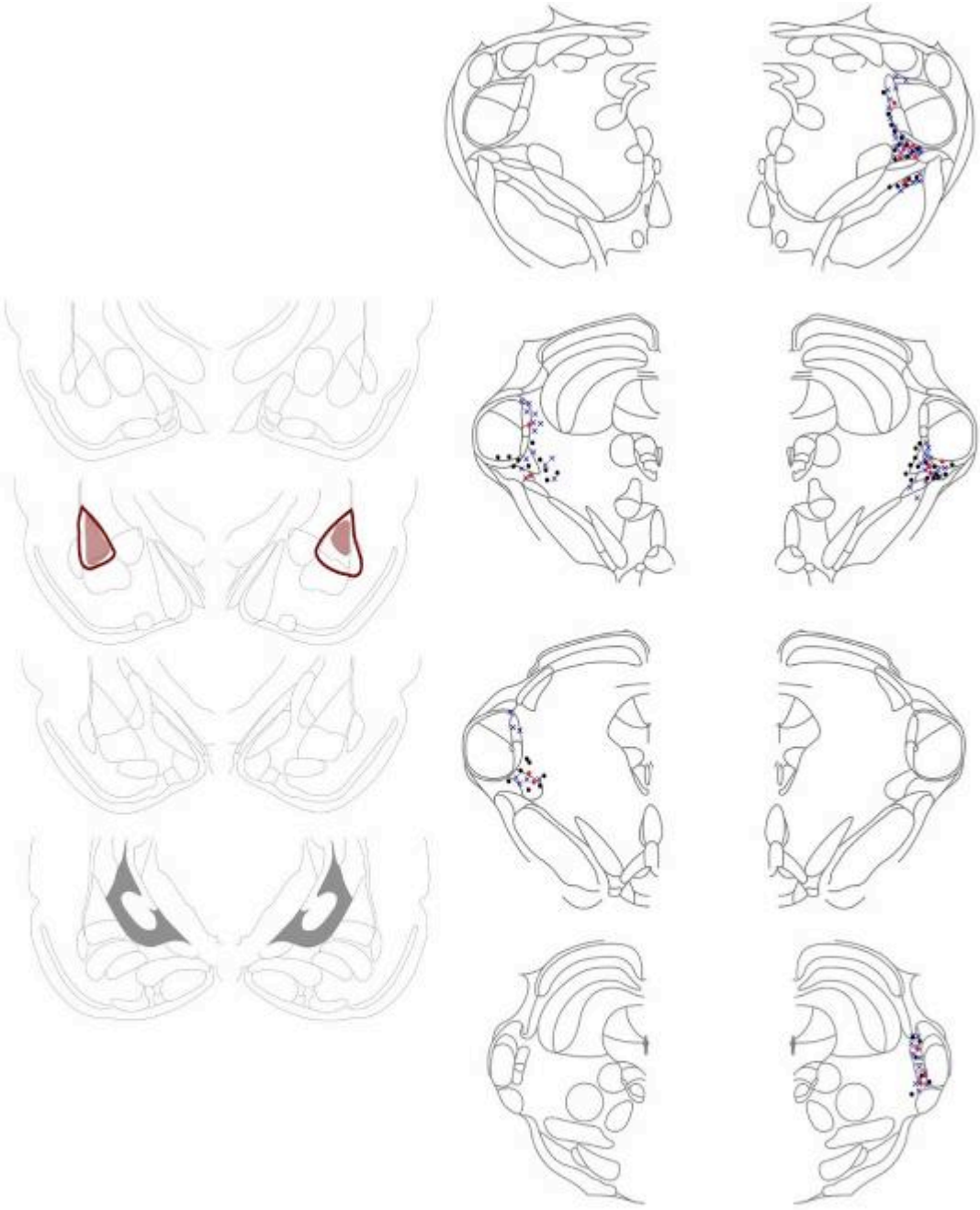

Figure 20b. Representative example of an Immediate shock animal. A) shows the diffusion of FG (largest and smallest) in the LA and B) the corresponding label in the auditory thalamus. (Blue cross = FG label, Black dot = EGR-1 label, Red dot = Double labelled cell)

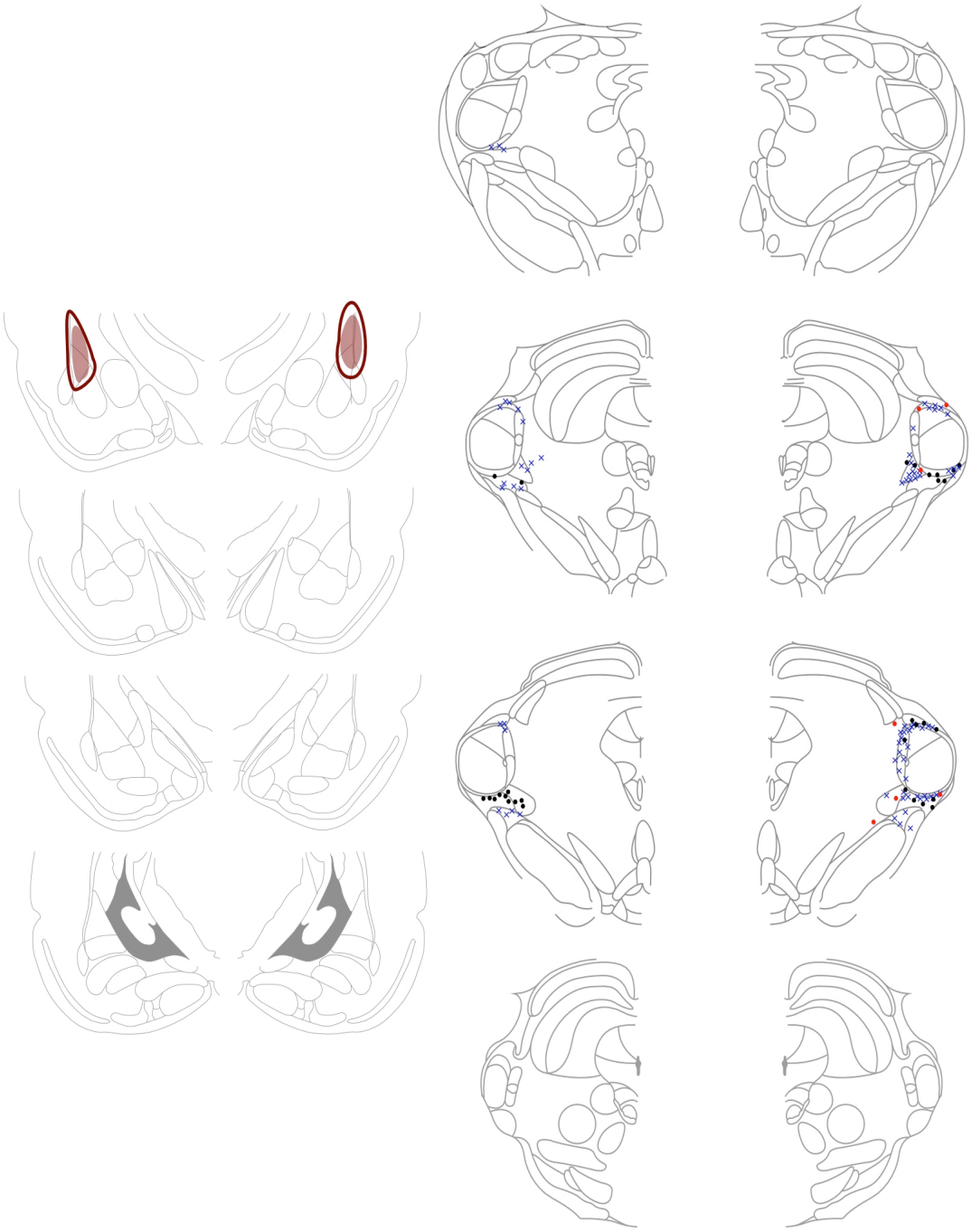

Figure 20c. Representative example of Naive animal. A) shows the diffusion of FG (largest and smallest) in the LA and B) the corresponding label in the auditory thalamus (Blue cross = FG label, Black dot = EGR-1 label, Red dot = Double labelled cell)

I. EGR-1 count across the auditory thalamus as a function of training

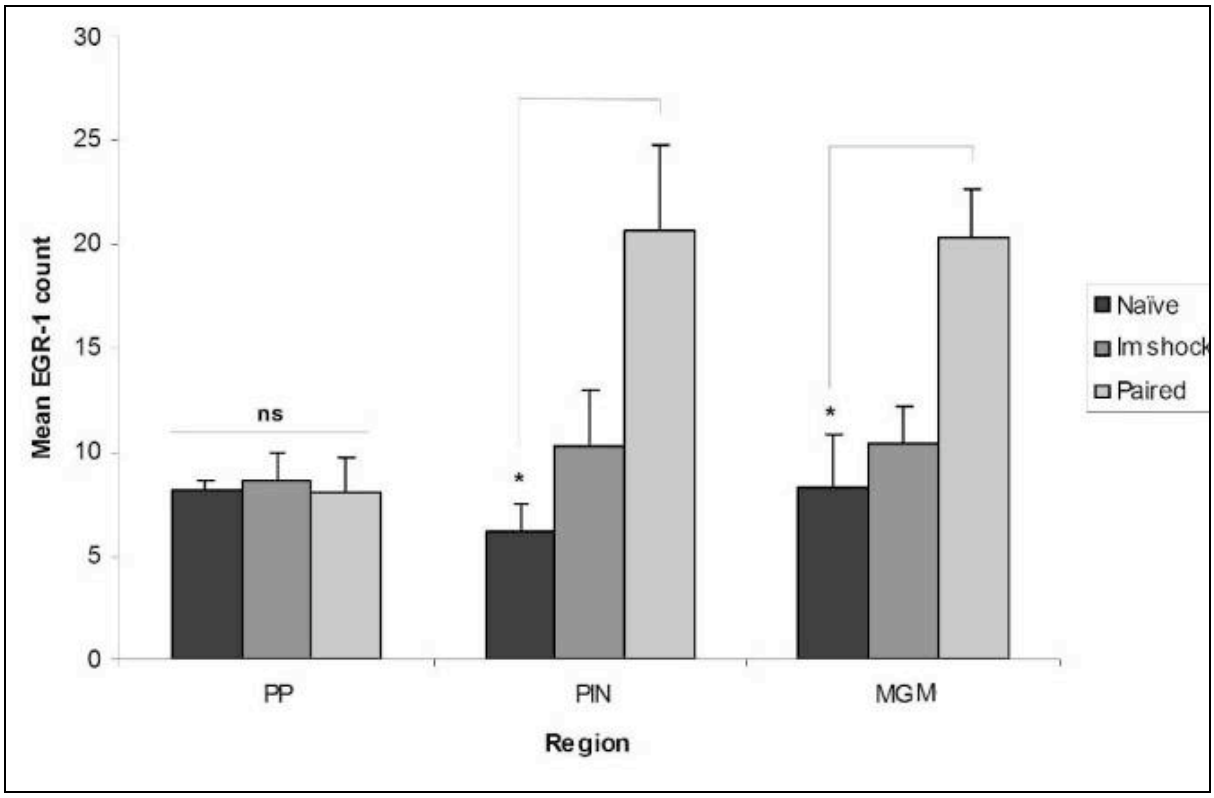

Figure 21. Mean (± SEM) EGR-1 cell count as a function of training across the three nuclei that comprise the auditory thalamus. Animals received intra-LA microinfusions of FG 10-14 days prior to training and were trained with either 3 tone shock parings, 3 immediate shocks, or functioned as naïve controls. Two hours post training brains were processed for the expression of EGR-1. *p<0.05.

Firstly an examination of whether EGR-1 upregulation in the auditory thalamus differed as a function of different training protocols' in animals who received intra LA microinfusions of FG was conducted. Thus, this EGR-1 label was counted irrespectively of FG label. Figure 21 shows the average EGR-1 count across the three structures' of the auditory thalamus. It shows that the number of EGR-1 positive cells were greater in the Paired group across both the PIN and MGM when compared to an Immediate shock and Naïve control. No differences were observed in the PP. One way ANOVA's revealed that the difference across the PIN was significant (F(2,14)=4.50,p<0.04) with Naïve animals differing significantly from Paired (p<0.05, Dunnett's t); the difference across the MGM was also significant

($F(2,13)=4.80, p<0.03$), again with Naive animals differing from the Paired ($p<0.05$, Dunnett's t). As expected no differences' were observed across the PP ($p=0.97$)

For further analysis EGR-1 counts across the nuclei that comprise the auditory thalamus were combined (see Figure 22). The graph shows that EGR-1 expression was greatest in the Paired group, followed by the Immediate Shock animals, while the Naïve controls exhibited the least labelling. A one-way ANOVA revealed that this difference was significant ($F(2,14)=5.63, p<0.02$) with the Paired animals differing significantly from both the Immediate shock and Naïve groups ($p<0.05$, Dunnett's t).

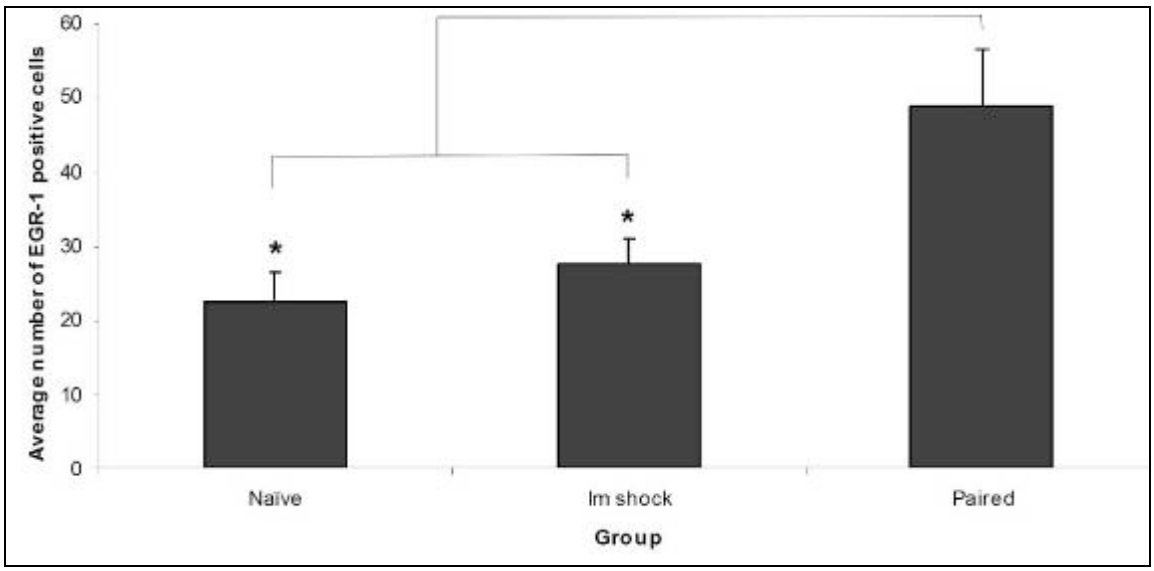

Figure 22. Mean (± SEM) EGR-1 cell count as a function of training across the auditory thalamus. Animals were trained with either 3 tone shock parings, 3 immediate shocks, or functioned as naïve controls. Two hours post training brains were processed for the expression of EGR-1

II. Fluorogold cell count within the auditory thalamus for each group

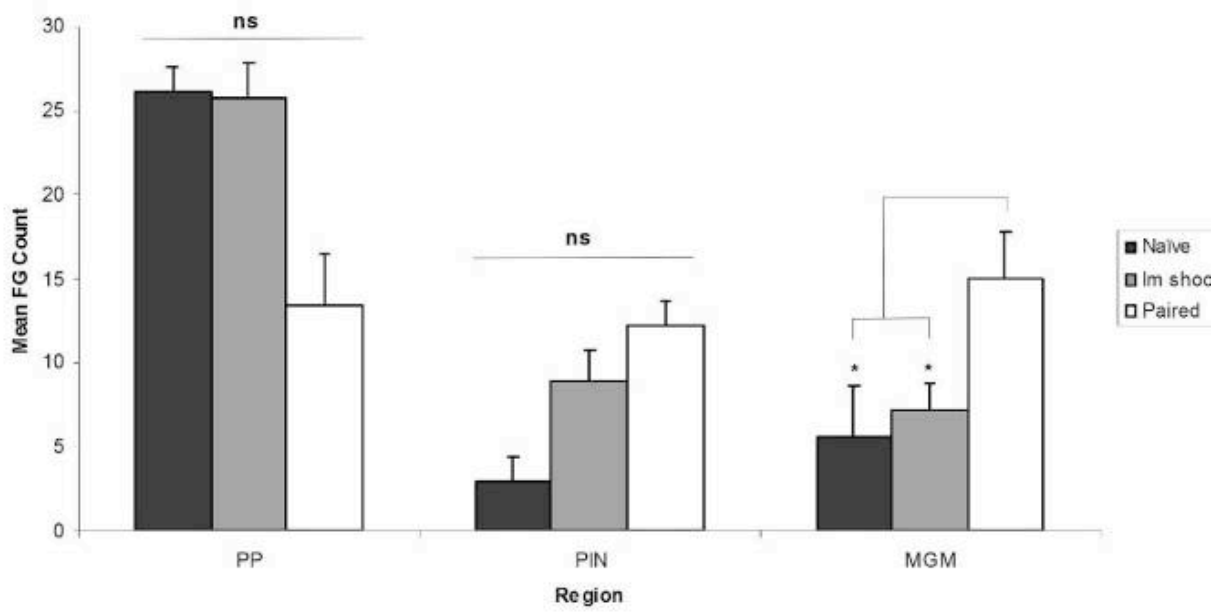

Figure 23. Mean (± SEM) fluorogold (FG) cell count across the nuclei that comprise the auditory thalamus. Animals received intra-LA microinfusions of FG 10-14 days prior to training and sacrifice.

In order to determine whether or not differences in FG diffusion within the LA and subsequent FG labelling in the auditory thalamus could confound the results a count of the number of FG labelled cells across the PP, PIN, and MGM was performed. Figure 23 shows the mean FG cells counts in the three nuclei of the thalamus of interest, it shows that there were some differences in FG label at a nuclei level. One way ANOVAs revealed no significant differences at the PP (p=0.45), and PIN (p=0.8). However, a significant difference across the MGM was observed (F(2,13)=7.45,p<0.007), with both the Naive and Immediate shock groups differing from the Paired (Dunnett's t p<0.05).

Figure 24 shows the FG count across the auditory thalamus as a whole. It shows that when the FG counts were quantified in such a manner there were no significant differences between groups. In line with this a one way ANOVA revealed no significant differences (p=0.48).

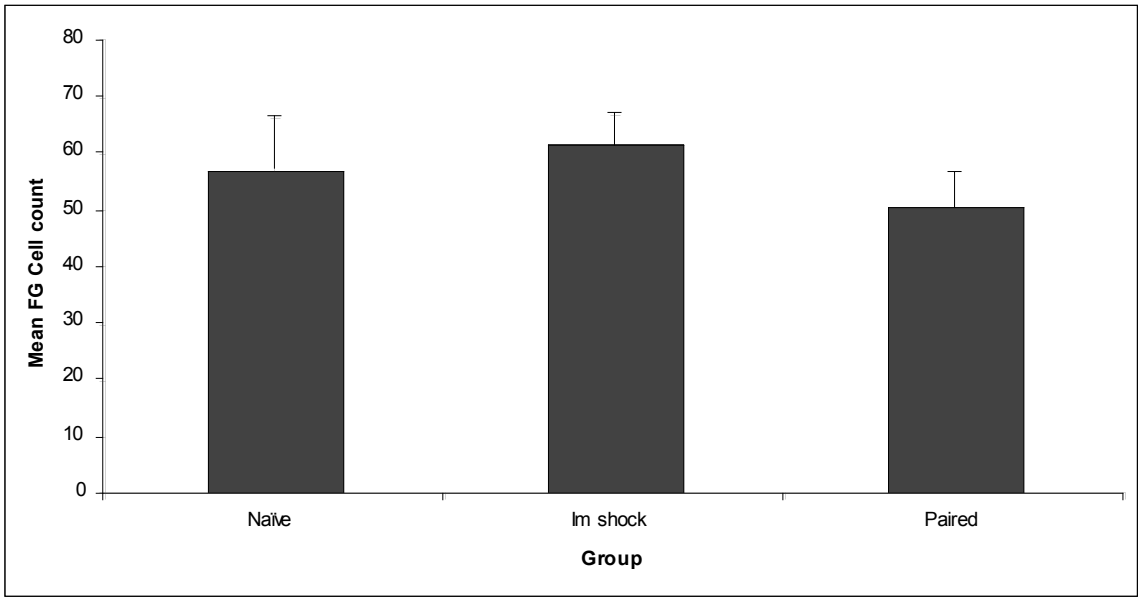

Figure 24. Mean (± SEM) fluorogold (FG) cell count across the auditory thalamus. Animals received intra-LA microinfusions of FG 10-14 days prior to training and sacrifice.

III. Percentage of FG positive auditory thalamic cells (LA projection cells) that are EGR-1 positive

The primary aim of this experiment was to examine differences in EGR-1 expression in auditory thalamic cells that constitute a component of the LA circuitry. To this end a quantification of the number of LA projection cells, or more specifically FG labelled cells that were also EGR-1 positive was conducted.

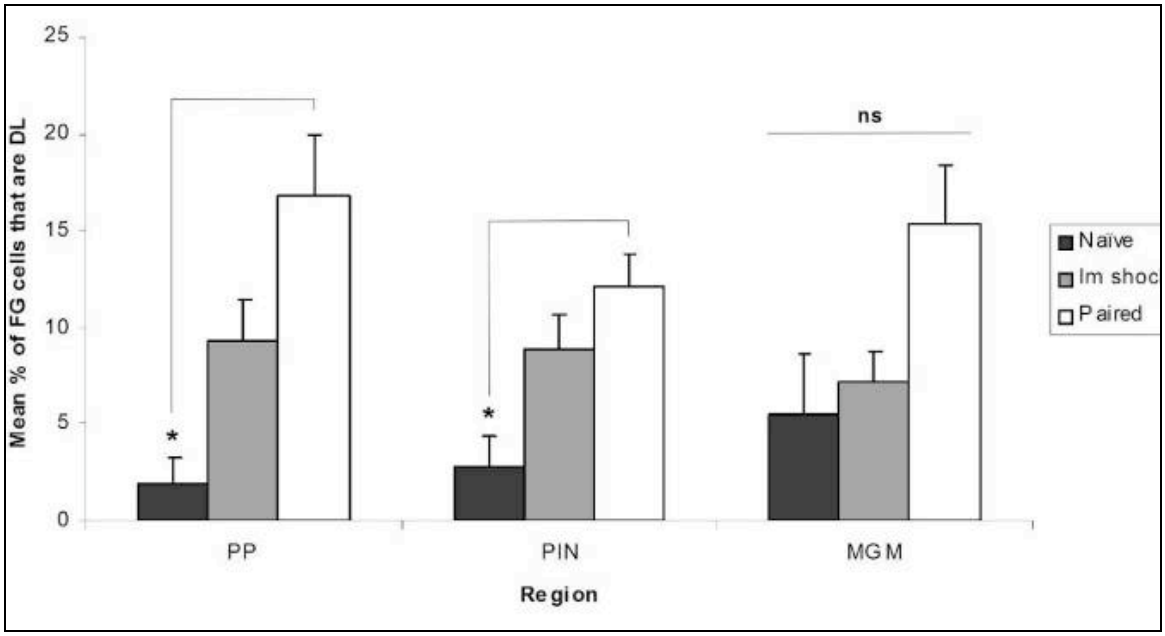

Figure 25. Mean percentage of fluorogold positive cells that are also EGR-1 positive (double labelled) as a function of training across the three nuclei that comprise the auditory thalamus. Animals' received intra-LA microinfusions of fluorogold 10-14 days prior to training. Animals were then trained with three tone shock pairings, three immediate shocks, or functioned as naïve controls. Two hours post training brains were analysed for the expression of EGR-1 and FG. *p<0.05.

Figure 25 shows the percentage of FG labelled cells in the auditory thalamus that were also EGR-1 positive, or more specifically double labelled. It shows that across all three nuclei the Paired animals exhibited a higher number of double labelled cells compared to both the Immediate Shock and Naïve animals. One way ANOVA's revealed significant differences across the PP (F(2,14)=7.46,p<0.006) with the Paired group differing from the Naives'(p<0.05, Dunnett's t). Differences were also observed across the PIN (F(2,14)=6.32,p<0.01) with the Paired group, again, differing from the Naïve (p<0.05, Dunnett's t). Group differences across the MGM approached significance (F(2,13)=3.74,p=0.052).

Consistent with previous analysis counts across the three nuclei that comprise the auditory thalamus were combined. The results are depicted in Figure 26 as seen the Paired animals exhibited a greater degree of double labelling than both Naïve controls and Immediate Shock animals. A one-way ANOVA revealed a significant difference

($F(2,14)=9.47$, $p<0.003$), with the paired group differing from both the Naïve controls and Immediate shock animals ($p<0.05$, Dunnett's t).

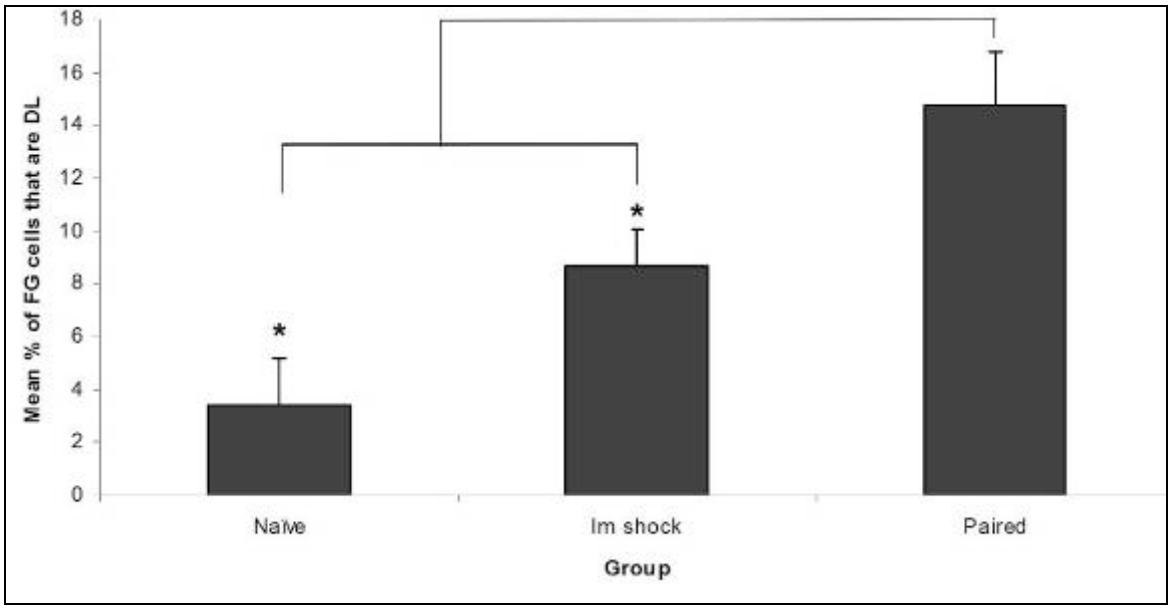

Figure 26. Mean percentage of fluorogold positive cells that are also EGR-1 positive (double labelled) as a function of training within the auditory thalamus. Animals' received intra-LA microinfusions of fluorogold 10-14 days prior to training. Animals were then trained with three tone shock pairings, three immediate shocks, or functioned as naïve controls. Two hours post training brains were analysed for the expression of EGR-1 and FG**.** *$p<0.01$

Discussion

The aim of this experiment was to determine the density of training induced EGR-1 auditory thalamic cells that are LA-projection cells. To this end, the retrograde tract tracer FG was microinfused into the LA at least ten days prior to training, animals were either trained with three tone shock pairings, three immediate shocks, or served as naive controls. Results indicated that paired presentations of a tone and footshock resulted in a significantly greater degree of EGR-1 labelling in the auditory thalamus when compared to both a Naive and Immediate Shock control. The results revealed no significant differences in the number of FG labelled cells as a function of training. However, a significant portion of EGR-1 upregulation in thalamic LA projection cells was observed in paired animals when compared to either an immediate-shock or naive control. Overall, my results indicate that conditioning results in a significant increase in

EGR-1 expression within auditory thalamic cells when compared to Immediate Shock and Naïve controls. Further, this pattern of regulation is repeated when quantification of EGR-1 regulation is restricted to auditory thalamic cells that project to the LA.

The number of double labelled cells and for that matter EGR-1 labelled cells in this experiment is a conservative estimate as can be expected in any double labelling study. More specifically, any inefficiencies in the labelling or visualization of label will reduce the number of double labelled cells observed more so than a single label study. With specific regard to detection of EGR-1 labelled cells an alkaline phosphatase detection method was chosen. This staining method produces a more diffuse and translucent stain than peroxidase substrates, meaning that while double labelled cells were reasonably easy to detect given the robust nuclear labelling enveloped within a red cell soma (specifically, the red phosphate cellular stain for FG) detection of EGR-1 positive cells by themselves proved to be a little more challenging. Thus, it is quite possible that a greater number of EGR-1 labelled cells were present within the auditory thalamus. Nonetheless, this does not impact on the percentage FG positive cells that were double labelled.

One of the reasons that the study was conducted was to improve the analysis conducted in Chapter 2 where EGR-1 upregulation as a function of five different training protocols was examined. In that particular experiment a significant difference between the Paired and Immediate Shock groups were not observed. Here in a replication of this experiment when EGR-1 count is done irrespectively of FG label the results reveal a significant difference between these two groups. However, as stated above the EGR-1 detection method used in this current experiment is not as sensitive as the phosphate substrate used in the research discussed in Chapter 2. Thus, the significant finding here could simply be a function of methodological differences. Most

importantly, however, when the results are restricted to LA projection cells, which as discussed are easier to quantify than EGR-1 on its own, a significant degree of EGR-1 upregulation is observed in the Paired group when compared to the other two controls.

As far as examination of the anatomic connectivity between the LA and the auditory thalamus my results are constant with a number of previous researchers who reported dense areas of LA projection cells localized within the PP/PIN, more so than the MGM. (LeDoux, 1990) In line with this, the density of training induced EGR-1 upregulation was also the greatest in the PP/PIN. Consistent with previous research the results did reveal some labelling in the dorsal region of the MG (MGD) (Doron, 1999, 2000). However, this is primarily a cortical projection area and may constitute a component of transynaptic circuitry (Doron, 2000). Given, the PP/PIN/MGM exhibited the most robust labelling the analysis was restricted to this region, and thus the MGD was disregard. Further, this kept the analysis consistent across the different experiments that comprise this thesis.

Researchers who have analyses the anatomical connectivity between the LA and auditory thalamus often restricted their analysis to excitatory neurons. It would have been advantageous to conduct a similar analysis in this current research, as the density of double labelled cells may have been greater. Indeed, it has been reported that around 50% or auditory thalamic LA projection cells are excitatory. The current results revealed that around 16% of all auditory thalamic projection cells were double labelled, if quantification had been restricted to glutamatergic-excitatory cells this percentage may have doubled. Nonetheless, the results of this experiment produced a significant result even thought cell type was not delineated.

Interestingly, auditory thalamic cells that project to the LA are also know to project to cortical auditory processing areas. Doron and colleagues (2000) microinfused

various retrograde tract tracers into cortical auditory association areas and the LA, they then quantified cells that project to each area in the auditory thalamus. Their results revealed that 43.7% of cells in the PP/PIN are double labelled cells, meaning that they project to both the LA and cortical auditory processing areas (Doron, 2000). More specifically, of all the LA projection cells 21.0% are double labelled (Doron, 2000). While such a finding does not directly reflect or lead to adjustment of my results, it does raise the possibility of joint regulation of gene expression in the auditory thalamus by both the cortex and the LA. Future research could be conducted to see the degree of retrograde regulation via these two brain areas.

Overall, the results of this research have revealed that within thalamic-LA projection cells a paired conditioning protocol results in a greater degree of EGR-1 upregulation when compared to an immediate-shock and naïve control. As a result within this experiment specific localization of gene expression in the auditory thalamic cells that are associated with the formation of a fear memory association has occurred, at least when compared to quantification of EGR-1 upregulation in the auditory thalamus as a whole. However, a very important point remains to be addressed. The results have shown that a significant degree of EGR-1 upregulation in the auditory thalamus as a function of acoustic fear conditioning. However, at this point the significance of this upregulation for auditory fear memory consolidation is unknown.

Chapter 5

Training-induced upregulation of thalamic EGR-1 is required for fear memory consolidation

Up to this point the research described in this thesis has examined EGR-1 modulation in the auditory thalamus at both a molecular and systems level. The research conducted for this dissertation has shown that EGR-1 protein is upregulated in the auditory thalamus as a result of training. This regulation is driven by the LA during the acquisition phase of fear memory formation as a result of NMDA receptor activation and NO synthesis. However, a key question remains regarding whether this presynaptic EGR-1 upregulation is necessary for the consolidation of a conditioned fear memory association. As previously discussed, it is possible that training induced EGR-1 upregulation could simply function to replenish the cell after periods of excitation. Alternatively, upregulation may be a perquisite for fear memory formation, whereby it primes the cells for memory formation, but this priming at a behavioural level is not sufficient for memory formation. However, there is research that has shown that EGR-1 upregulation during memory formation is not simply correlated with cellular excitation and serves a significant role in memory formation.

The functional role of EGR-1 in memory consolidation

Most research concerning the functional role of EGR-1 in memory has involved the use of EGR-1 knock out mutant mice (specifically, EGR-1 −/− homozygous, where there is completely knock out of EGR-1 expression; or EGR-1 +/− heterozygous, where theoretically there is a 50% knock down of EGR-1 expression). For example, Jones and colleagues (2001) used homozygous mutants' to examine whether EGR-1 expression is pertinent for hippocampal dependent learning tasks. Importantly, these animals

exhibited no gross hippocampal abnormalities, basal synaptic transmission, and short-term plasticity were also within normal ranges. For example, assessment of working memory using the T-Maze revealed intact functioning. Short-term memory on tasks such as the social transmission of food preference task and object recognition were also normal. However, when memory consolidation was assessed deficits became apparent, this was true for the social transmission of food preference, object recognition, and conditioned taste aversion. On the other hand, when the homozygous mutants were assessed on their spatial navigation skills using the water maze task they exhibited acquisition deficits and LTM deficits (Jones, 2001). Overall, the authors argued that their results demonstrated that EGR-1 is typically necessary for the transition of memories from a short-term to a long-term state.

The researchers then turned their attention to heterozygous mice who exhibited EGR-1 mRNA levels that were approximately half those observed in wild-type controls. Their performance on learning and memory tasks was slightly more variable than the homozygous mice. They exhibited similar levels of impairment in spatial navigation. However, the deficits of the heterozygous mice were less pronounced when assessed on taste aversion and social transmission of food preference specifically, their performance on these tasks fell in between wild type and homozygous mice. In addition, no deficit was observed on the novel object recognition task. Therefore, the results indicate that different degrees of EGR-1 reduction differentially impair performance on different memory tasks.

In a subsequent study, Bozon and colleagues (2002) modified the object recognition task so that they could compare memory performance across homozygous, heterozygous, and wild types for both object recognition and memory for the spatial location of the objects, thereby increasing the memory demands. In this series of

experiments they used three objects rather than two, as employed in the Jones (2001) study described above. On the object recognition component of the task both the wild type and heterozygous mice exhibited intact LTM. Consistent with the previous research the homozygous mice showed a significant level of impairment in the second phase of the experiment where spatial location of one of the familiar objects was changed. Again, wild type mice demonstrated intact LTM as they spent a significant period of time exploring the object in the novel position. Consistent with the impairment on the water maze task, both heterozygous and homozygous EGR-1 mutants exhibited equivalent deficits in long-term spatial memory as their time spent exploring the familiar object in the novel position was at chance. Thus, the results of this research showed that memory for objects and the spatial location of objects is differentially affected by a 50% reduction in gene expression under high task demands. That is, while the heterozygous animals could consolidate and recall information relating to basic characteristics of the object, they were not able to consolidate information concerning the spatial location of the object. The important point raised by this research is that there is not a simple relationship between levels of EGR-1 protein upregulation and behavioural performance. The authors go onto suggest that a minimum threshold of activation of EGR-1 is necessary for the stabilization of a memory trace that may be task dependent, whereby the 50% reduction in EGR-1 expression observed in the heterozygous group is still below the threshold required for spatial memory formation (Bozon, 2002; Jones, 2001).

With regard to the fear system, research has shown that a modest 11% reduction in EGR-1 protein levels in the LA is sufficient to impair auditory fear memory association (Malkani, 2004). However, in this series of experiments an antisense oligonucleotide (ODN) was used to block EGR-1 protein upregulation. The antisense

binds to complementary EGR-1 mRNA preventing it from being translated into a protein. As a result, this manipulation only interferes with EGR-1 upregulated as a function of training, while basal EGR-1 levels necessary for normal functioning remain unaffected. The authors reported impaired auditory fear memory consolidation assessed 24 hours after conditioning while STM and post-shock reactivity was left intact, indicating memory acquisition and normal behavioural processing of the footshock. An important point raised by this experiment is the fact that an 11% reduction in EGR-1 protein levels was sufficient to impair memory consolidation. This supports the idea that some degree of EGR-1 upregulation is not directly related to memory consolidation and that EGR-1 protein levels appear to need to reach a threshold for memory formation to occur. Furthermore, the threshold appears to be more stringent for amygdala based fear memory formation than the hippocampal tasks described above. Indeed, a lower threshold may be maladaptive, meaning that the formation of a fear memory would occur more readily and inappropriately.

This current experiment

In this series of experiments an EGR-1 ODN was used to knock down EGR-1 expression in the auditory thalamus. As briefly mentioned above, an antisense ODN binds to the complementary (sense) mRNA thus preventing ribosomes' from translating the message into the corresponding protein, thus effectively turning the gene off. A 250pmol concentration of EGR-1 ODN was utilized, the same concentration as that used by Malkani and colleagues (2004). In their experiments they demonstrated that blockade of EGR-1 protein upregulation in the LA prevents contextual fear memory consolidation. In this experiment the affect of EGR-1 knockdown in the auditory thalamus on auditory fear memory consolidation was assessed. It is expected that EGR-1 upregulation in the auditory thalamus is required for fear memory consolidation. As a

result it is hypothesised that a significant impairment in LTM fear memory consolidation will be observed in animals who receive intra-LA EGR-1 ODN microinfusions compared to animals that receive microinfusions of scrambled-ODN-control.

Method

Experiment one: The affect of EGR-1 knockdown in the auditory thalamus on the consolidation of auditory conditioned fear

Subjects

Thirteen adult (300-350g) male Sprague Dawley rats (Hilltop Laboratories, Philadelphia, PA) served as subjects. They were housed individually in plexiglass cages in a vivarium maintained on a 12 h light/dark cycle. All procedures were conducted during the light phase of the cycle. Food was available *ad libitum* throughout the experiment.

Surgery

Surgery protocols were identical to those described in previous chapters. The cannulae for this experiment, however, were 26 gauge (Plastics One, Roanoke, VA) and were aimed at the auditory thalamus (AP: 5.5, ML: +2.8, DV: -6.6).

Oligodeoxynucleotide design and preparation.

EGR-1 antisense ODNs were designed under guidelines described in Malkiani et al. (2004). Briefly, scrambled and antisense ODNs (Midland Certified Reagent Company, Midland, Texas) were obtained that encoded antisense and scrambled sequence for the EGR-1 mRNA sequence. The scrambled ODNs served as controls and did not show significant homology to sequences in the GenBank database. The following sequences were used: 5'-GGTAGTTGTCCATGGTGG-3' (antisense) and 5'-

GTTGGAGTCGGTGGTTCA-3' (scrambled). EGR-1 antisense ODN and its scrambled-antisense control, were dissolved in physiological saline to yield 250pM / 0.5µl concentrations. Both drugs were infused bilaterally into the auditory thalamus at a rate of 4min/µl.

Behavioural procedures

The day before training the animals were habituated to handling and cannula insertion. On training day, animals were randomly divided into Antisense-ODN (*n*=5) and Scrambled-control (*n*=8) groups. Rats were then handheld, dummy cannulas removed and infusion cannulae which protruded 2mm below the tip of the guides were inserted. The appropriate drug was then bilaterally infused. After infusion was complete the cannulae were retained in the guide for a further 2min to allow drug diffusion from the tip.

Ninety minutes following drug infusion the animals were placed in the conditioning chambers and allowed three minutes to acclimate. They were then presented with one tone-shock paring. The tone (5 kHz, 75 dB) was presented for 30 seconds and co-terminated with a one second 1.5mA footshock.

- *Short-term-memory test*

Three hours post training the animals' STM was assessed. They were returned to the training chambers. However, some of the contextual cues were changed. Specifically, the grid floor was covered with black perspex and a peppermint scent was introduced to the environment. Testing consisted of three 30-sec tone presentations, identical to those used in the conditioning trial, at an ISI of 150sec.

- *Long-term-memory test*

Twenty-four hours after training the animals' LTM was assessed. They were once again returned to the conditioning chambers and the new contextual cues that were present during the STM test were retained. In addition however, the LTM test was performed in complete darkness. During the test the animals were presented with 5 tone presentations (again, 30s, 5 kHz, 75 dB) at an ISI of 150sec. See Figure 27 for a schematic of the behavioural protocols used in this experiment.

Data analysis

For each animal, during both the STM and LTM tests the total time (in sec) of freezing during each tone presentation was calculated and then expressed as a percentage of behavioural freezing. This was then averaged to give an overall percentage freezing response for each animal. A group average was then determined. All data was analyzed using a repeated measures ANOVA and *post-hoc t*-tests where appropriate. Animals that exhibited behavioural freezing below 80% in the short-term memory test (n=3) were excluded from the analysis.

During training movement amplitudes 20 sec prior to the 1-sec-shock presentation and 20 sec post-shock presentation were recorded. Total seconds of freezing during each time period were recorded and expressed as a percentage of behavioural freezing. Results were compared using *t*-tests.

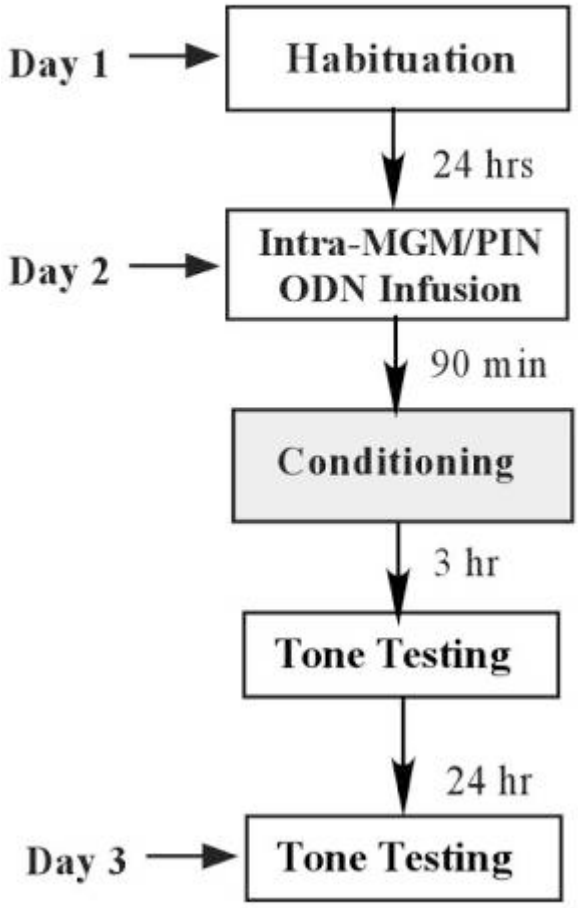

Figure 27. Behavioural procedures used to examine STM and LTM memory impairment after intra-thalamic microinfusions of EGR-1-ODN. The animals were habituated to handling and cannulae removal the day prior to training. The animals received intra-thalamic infusions of ODN 90-min prior to training, animals were then conditioned. Three hours post conditioning the animals STM was assessed, 24 hrs after conditioning their LTM was assessed.

Cannula placement verification

After the memory tests were complete animals were euthanized with an overdose of chlorohydrate (25% solution) and transcardially perfused with 50 ml of physiological saline followed by 250 ml of 10% buffered formalin. The brains were promptly removed and postfixed in formalin for approximately 24hours; they were then transferred to a long term 20% glycerol-0.1 M PB cryoprotectant solution until sectioning.

Histology

Forty micron sections of the auditory thalamus were taken. These were stained with cresyl-violet and the location of the cannulas within the auditory thalamus determined using light microscopy.

Experiment 2: Diffusion of biotin EGR-1 antisense in the auditory thalamus

Subjects

Six animals that were used for the EGR-1 antisense experiment were reused to determine the diffusion radius of microinfusions of 250pM / 0.5µl biotinylated-EGR-1 antisense.

Experimental procedures

The rats received a bilateral infusion of the biotinylated-EGR1-antisense ODN in exactly the same concentration (250-pmol) and manner as described above for the EGR-1-antisense-ODN in the previous experiment. They were euthanized with chlorohydrate (250 mg/kg, IP) 30 min (n = 2), 1hr (n = 2), or 3hr (n = 2) after completion of drug infusion.

The animals were then transcardially perfused with ~50ml PBS followed by 250ml of ice cold 4% paraformaldehyde in 0.1 M PB. Brains were postfixed in 4% paraformaldehyde-PB solution overnight, then transferred into a 20% glycerol-0.1 PB cryoprotectant solution and stored at 4 ºC until sectioning.

Forty-micron free-floating sections of the auditory thalamus were taken using a sliding microtome. Sections were firstly, washed in PBS and then incubated in a 3% hydrogen peroxide solution for 20mins in order to sequester any hemoglobin that may be present. They were then transferred to a standard ABC solution for one hour, washed

in PBS, and then transferred to a DAB peroxidase solution for developing. Sections were washed and mounted on slides and stained with crysal-violet and the diffusion dynamic of the drug determined.

Results

Histology

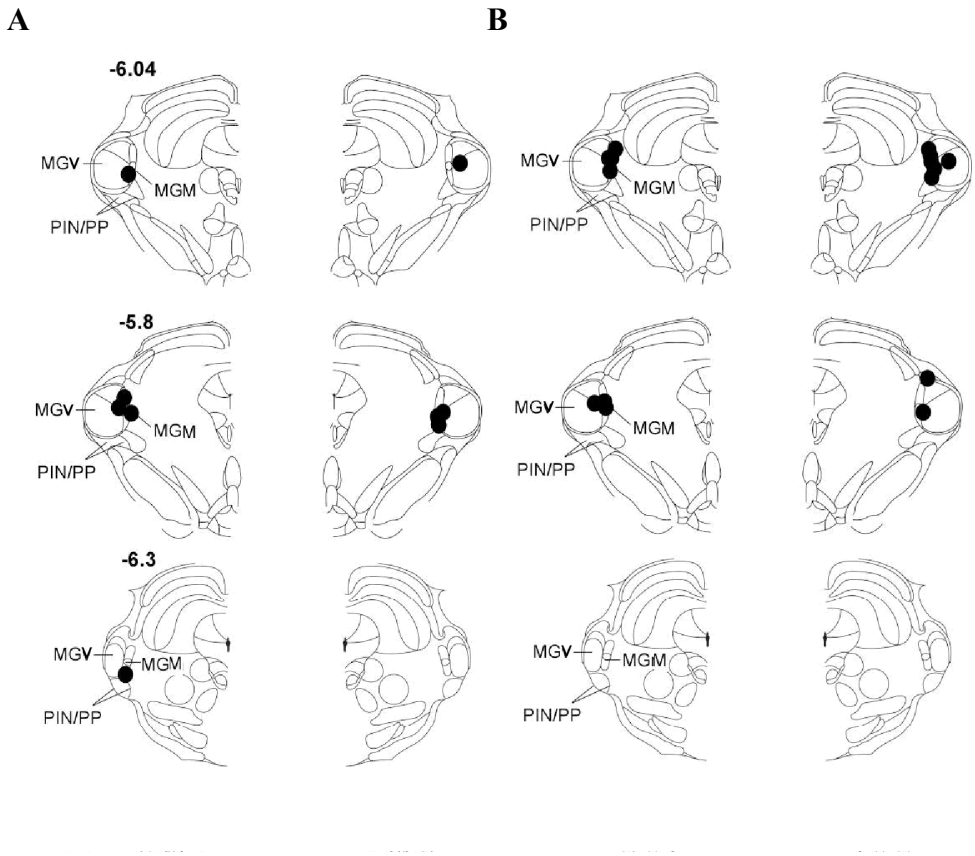

Figure 28. Schematics of the auditory thalamus depicting cannula placements for A) Antisense and B) Scrambled drug groups. Schematics' were adopted from the rat brain atlas (Paxinos, 2004). MGV: ventral region of the medial geniculate, MGM; medial region of the medial geniculate, PIN: posterior intralaminar nucleus, PP peripeduncular nucleus.

Figure 28 depicts the intra-auditory thalamic cannula sites for antisense-ODN and scrambled-ODN animals. All placements are superimposed on coronal sections taken from the rat brain atlas (Paxinos, 2004). As illustrated cannula tips were predominantly located in the MGM region of the thalamus.

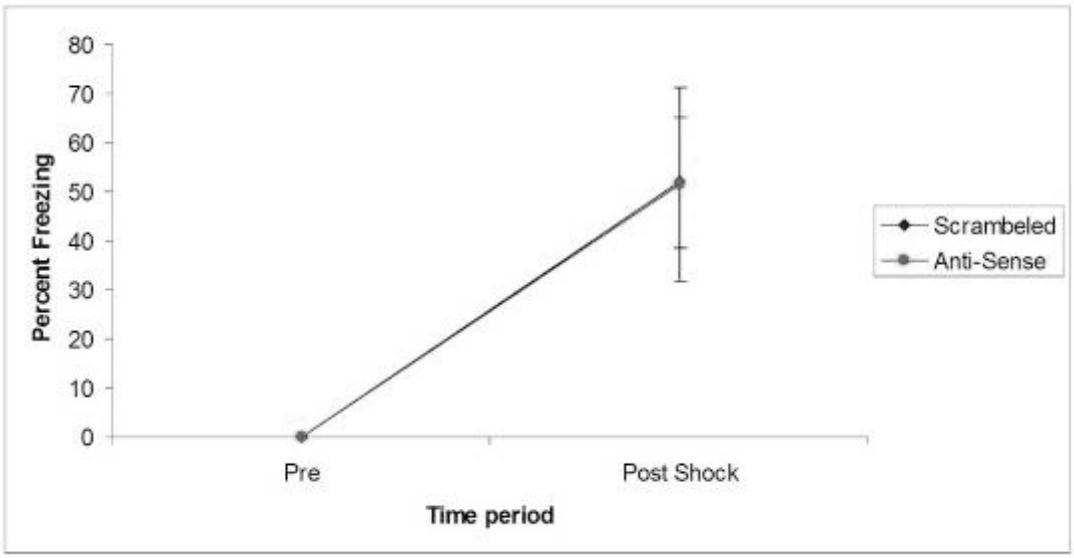

Figure 29. Average percent freezing (± SEM) pre and post shock as a function of bilateral intra auditory thalamic microinfusions of 250-pmol EGR-1 antisense ODN (*n*=5) or 250-pmol EGR-1 scrambled ODN (*n*=8).

Figure 29 depicts the average percent of freezing for the scrambled control and EGR-1 antisense ODN groups 20 sec prior to the 1-sec footshock presentation (Pre) and 20 sec following termination of the footshock-US (Postshock). The results indicate that both groups had equivalent and intact levels of post-shock freezing, indicating normal processing of stimuli.

Long-term memory after EGR-1 knockdown in the auditory thalamus

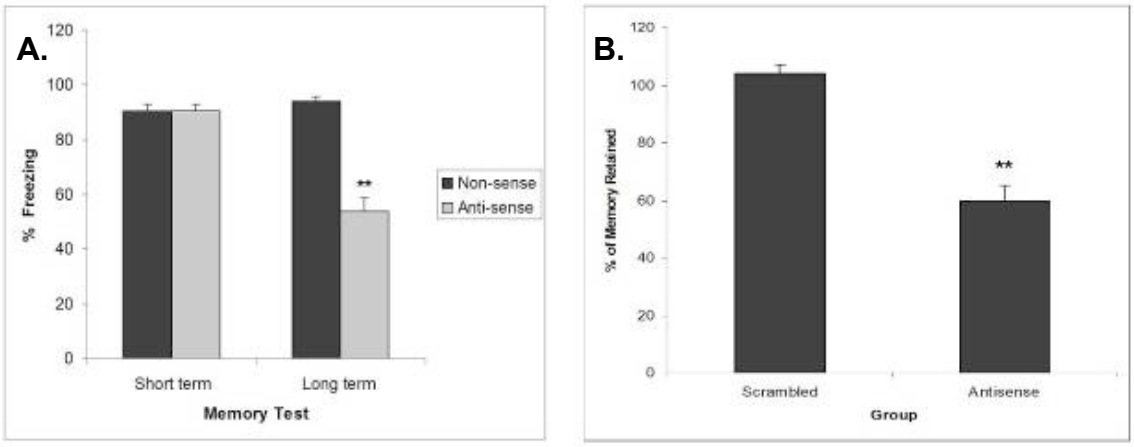

Figure 30. Memory impairment induced by EGR-1 knockdown in the auditory thalamus. A) Mean percent freezing (+SEM) for both the short term (3 hr post training) and long-term (24 hrs post training) memory. Animals' received intra-thalamic microinfusions of EGR-1 antisense ODN (n=5) or EGR-1 scrambled ODN (n=8) (both at 250-pmol) prior to one tone-shock conditioning trial. B) Depicts the mean percentage (+SEM) of LTM retained relative to STM.

Figure 30 depicts both STM and LTM test for animals infused with either scrambled or EGR-1 antisense ODN. The results revealed that intra-thalamic microinfusions of EGR-1 antisense ODN left STM, assessed 3 hr post training, intact while, LTM assessed 24 hr post training was impaired relative to scrambled controls. A 2(Memory Test: Short-Term vs. Long-Term) X2 (Group: Non-Sense vs. Anti-Sense) repeated measures ANOVA, with the Memory Test factor as the repeated measure yielded a significant main effect of Memory Test ($F(1,11)=38.10, p<0.001$), a significant main effect of Group ($F(1,11) =46.52, p<0.001$) and a significant Memory Test X Group interaction ($F(1,11)=53.51, p<0.001$). For further analysis, the amplitude change during the LTM test was expressed as a percentage of that during the STM test for each animal (Figure 30B). Relative to controls the ODN-EGR-1 group exhibited impaired LTM memory retention ($t(11)=-6.94, p<0.001$). Collectively, these results indicate that knockdown of EGR-1 protein in the auditory thalamus significantly impaired fear memory consolidation while leaving STM or the acquisition of auditory conditioned fear intact.

Diffusion of biotinylated EGR-1 antisense ODN in the Auditory Thalamus

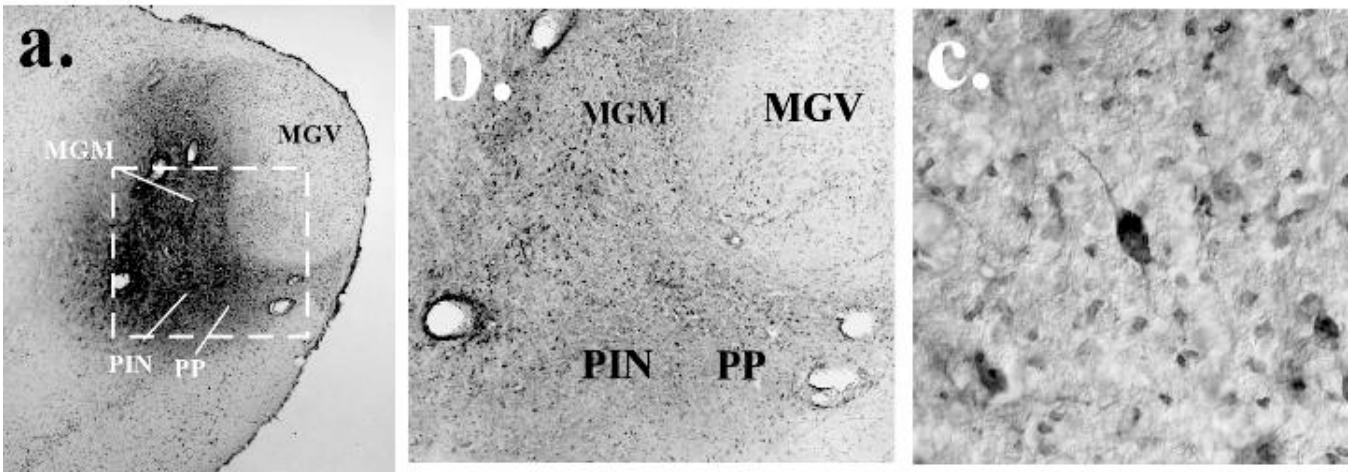

Figure 31. Diffusion dynamics of EGR-1 antisense ODN 30min post infusion into the auditory thalamus. The micrograph A. illustrates that the diffusion of the drug was localized to the PP, PIN and MGM; the auditory thalamus. Micrographs B and C are successively higher magnifications showing that the label was successfully taken up by the cell bodies.

Figure 31 depicts the diffusion of biotinylated EGR-1 antisense ODN 30 min post infusion at three successive magnifications of the auditory thalamus. My results indicated that the ODN was remarkably confined to the PP, PIN, MGM while uptake into the MGV, the thalamocortical relay nucleus that projects to the primary auditory cortex (Romanski, 1993; Shi, 1997), was not apparent. Examination of biotinylated EGR-1 antisense ODN diffusion at a higher magnification revealed that the ODN was localized to cell bodies, indicating active cellular uptake of the ODN. Labelling of the biotinylated EGR-1 ODN was less apparent 3 hr post infusion indicating complete cellular uptake (data not shown).

Discussion

The previous results had shown that auditory fear conditioning increases EGR-1 protein levels in the auditory thalamus. The aim of this experiment was to determine whether this increase in EGR-1 was necessary for auditory fear memory consolidation.

Intra-thalamic microinfusions of an EGR-1 ODN were employed to prevent training induced EGR-1 upregulation. A 250-pmole concentration of EGR-1 antisense-ODN was selected based on preliminary investigations and previous research by Malkani and colleagues (2004), who reported an 11% decrease in EGR-1 protein after intra LA microinfusions at the same dosage. Pre-training intra-thalamic microinfusions of EGR-1 ODN prevented the consolidation of a fear memory association assessed 24 hours after training. Whereas, both post-shock freezing and STM, assessed three hours post training, were left intact. These results indicated that animals with EGR-1 knockdown exhibited normal emotional processing during the conditioning procedure and transient STM formation.

In this series of experiments the animals that received intra-thalamic EGR-1-ODN microinfusions were not retrained. Researchers often include this subsequent step as it proves that the drug manipulations are responsible for the memory deficits and that the animals were indeed able to form a fear memory association, and that gross mechanical manipulations, specifically intra-thalamic cannula implantation, were not responsible for the behavioural deficit observed in the antisense-ODN group. Results did, however, reveal robust STM memory formation in the antisense group, indicating intact fear memory processing. Thus, while this additional step in my experimental procedures may have eliminated the likelihood that thalamic cannula implantation caused the LTM memory deficit, it seems that this possibility may be unlikely. Thus, this series of experiments points to the fact that EGR-1 protein knockdown in the auditory thalamus impairs auditory conditioning.

Our findings extend previous research concerning the fear system in which EGR-1 upregulation in the LA was shown to be required for auditory fear memory consolidation (Malkani, 2004). However, given the earlier research findings detailed in

this thesis, whereby the LA was shown to modulate thalamic EGR-1 expression, the results of this study have specifically shown that EGR-1 upregulation is necessary for presynaptic aspects of auditory fear memory consolation. In addition, Malkani and colleagues (2004) reported an 11% decrease in LA EGR-1 protein levels after infusion of an equivalent dosage of EGR-1-ODN as employed in this current study. It would have been interesting to complete a similar examination whereby EGR-1 protein knockdown in the auditory thalamus after a 250 pM EGR-1-ODN microinfusions was examined. Perhaps, a different percentage of EGR-1 knockdown would be observed.

As discussed previously there is some debate as to whether the EGR-1 upregulated as a result of conditioning is specifically related to cellular processes associated with memory formation, or other functional possibilities' not directly related to the formation of a fear memory. For example, 1) EGR-1 protein upregulation may simply be a function of stimuli novelty, priming the cell for memory consolidation; or 2) upregulated EGR-1 may serve to replenish intracellular machinery after a significant level of excitation or metabolic demand (Knapska, 2004). It is in fact possible that the EGR-1 knockdown in this study interfered with EGR-1 required for these processes. Given that the ODN concentration employed in this study is thought to produce a mild decrease in EGR-1 protein (~11%) and the fact STM was intact, it is plausible that processing of stimuli was normal. Suggesting that EGR-1 upregulated in response to novelty may have been within the normal biological range. It, therefore, appears that during this experiment EGR-1 upregulation pertinent for fear memory consolidation was prevented. That is, on a biological level it could be argued that it is easier to prime a cell for memory consolidation due to exposure to stimulus novelty than for actually consolidation to occur. Furthermore, it is likely that a minimum threshold of EGR-1 upregulation is necessary for the stabilization of a memory trace (Bozon, 2002). Given

that memory consolidation needs to be tightly regulated, so that only pertinent information is stored, it stands to reason that learning-induced EGR-1 upregulation is more sensitive to antisense-ODN knockdown than other more routine cellular processes. In line with this, there is the question of whether the EGR-1 knockdown in this experiment interfered with replenishment type processes in the cell, leaving the implicated cells in a compromised condition so that their normal functioning was impaired. However, STM assessed 3 hours post conditioning was intact, therefore suggesting normal if not enhanced cellular responding within the network.

EGR-1 is a transient transcription factor known to play a role in memory consolidation. My results suggest that EGR-1 contributes to presynaptic orchestration of cellular modifications that complement those that occur postsynaptically in the LA during fear memory consolidation. At this point it is important to readdress previous research that has illustrated that, while the auditory thalamus receives both US and CS associated neurotransmission (Bordi, 1994) and that plasticity in the auditory thalamus is required for fear memory consolidation (Apergis-Schoute, 2005), it is, however, not sufficient for its formation (Schafe, 2005). Instead, the LA appears to be the pivotal anatomical location of the fear memory association. Indeed, it has been suggested that associative tone-footshock activity in the auditory thalamus contributes to a LA-based fear memory representation by fine tuning inputs (Bordi, 1994). However, based on my findings up to this point, it seems reasonable to assume that EGR-1 upregulation in the auditory thalamus could contribute, in part, to the formation of a LA-based fear memory representation via modification of auditory thalamic cell terminals within the LA. That is, plasticity in the auditory thalamus is required for fear memory consolidation but in a manner that supports the formation of an LA based fear memory representation. In line with this, previous research has revealed that EGR-1

upregulation serves to modulate, or is associated with an increase in the expression of presynaptic proteins, specifically synapsin I and II and synaptophysin (Matsubara, 2001; Petersohn, 1995; Rossino, 1995; Theil, 1994). Thus, it stands to reason that training induced EGR-1 upregulation in the auditory thalamus is required, in part, for fear memory consolidation because of its ability to regulate cellular proteins that could potentiate auditory thalamic input to the LA.

Chapter 6

Training induced thalamic EGR-1 regulates conditioning-induced increases of the presynaptic proteins synapsin I and II; and synaptophysin

The research in this dissertation has revealed presynaptic, or LA based, regulation or presynaptic signalling pathways in the auditory thalamus. More specifically, results have indicated that the LA drives EGR-1 protein upregulation in auditory thalamic cells that project to the LA and that this upregulation is obligatory for auditory fear memory consolidation. Previous research has shown that activation of the ERK signalling in the auditory thalamus is necessary for the development of conditioned induced plasticity in the LA (Schafe, 2005). Such a result indicates that activation of intracellular pathways in the auditory thalamus serves to support the formation of an LA based fear memory representation. Further, unpublished observations from our lab have revealed that ERK activation in the auditory thalamus is required for training induced increases in thalamic EGR-1. Suggesting that EGR-1 upregulation in the auditory thalamus could be required for the development of an LA-based fear memory representation by potentiation presynaptic functioning. As discussed previously EGR-1 is an IEG that, in turn, acts as a transcription factor. This means that once the protein is translated it can return to the nucleus where it can regulate the expression of late response genes. Such genes could include those associated with proteins that play a key role in presynaptic functioning.

Presynaptic mediation of memory formation

As mentioned in the introduction potentiation of neurotransmitter release at the presynaptic membrane is thought to assist in an increase in synaptic efficacy necessary for memory formation (Krueger, 2006). There are two models of sustained alterations in neurotransmitter release that may underpin presynaptic potentiation. The first

involves an increase in the fusion pore size and an increase in the frequency of the formation of a fusion pore. The second involves an increase in the probability of neurotransmitter release. Thus, the readily releasable pool of synaptic vesicles are increased.

Below the presynaptic proteins the synapsins and synaptophysin are introduced, these presynaptic proteins are capable of mediating either of the above mechanisms of presynaptic potentiation. Therefore, functionally, an increase in the density of either protein would signify the development of a more efficient presynaptic terminal. Most importantly the synapsins and synaptophysin are known to be downstream of EGR-1 upregulated or correlated with its upregulation respectively (Matsubara, 2001; Petersohn, 1995; Rossino, 1995; Theil, 1994).

The synapsins

The synapsins are divided into two isoforms: synapsins I and II, both of which are regulated by EGR-1 (Petersohn, 1995; Theil, 1994). These presynaptic proteins colocalize with synaptic vesicles and are known to play a role in the regulation of neurotransmitter release, axonal elongation and the formation and maintenance of synaptic contacts (Huttner, 1983). Research has shown that synapsin I and II are membrane bound and specifically localized to nerve terminals' (De Camilli, 1990). Moreover, synapsin I and II are found tightly attached to synaptic vesicles at these terminals (De Camilli, 1990). As well as localizing with vesicles the synapsin also interact with the actin filaments' of the cytoskeleton in this manner they act to cross-link synaptic vesicles to the cytoskeleton of the neuronal terminal (Benfenati, 1989).

Functionally, the role synapsins play in presynaptic physiology appears to be diverse. In a dephosphorylated state the synapsins are bound to the cytoplasmic surface

of synaptic vesicles inhibiting these vesicles from interacting with the plasma membrane (Ceccaldi, 1995). An increase in intracellular calcium at nerve terminals ultimately leads to phosphorylation of the synapsins, which in turn leads to dissociation of the protein from the vesicle and neurotransmitter release (Greengard, 1987). However, antagonizing the activity of the synapsins slows vesicle release kinetics at the active zone (Hilfiker, 1999), suggesting that the synapsins play a role in vesicle fusions with the membrane and thus a more direct role in vesicle release at the active zones of presynaptic terminals. Moreover, it has been suggested that synapsins play a role in regulating neurotransmitter release by controlling the number of vesicles available for fusion (De Camilli, 1990). For example, microinfusions of an antibody for the synapsins into lamprey reticulospinal axon results in selective loss of distal reserve pool of vesicles located away from the plasma membrane (Pieribone, 1995). This distil pool is thought to contain neurotransmitter vesicles utilized during periods of high stimulation.

Synaptophysin

Due to its interaction with vesicle-SNARE proteins is believed to play a role in the formation of a fusion pore and thus fusion and exocytosis of neurotransmitter (Valtorta, 2004). More specifically, synaptophysin interacts with synaptobrevin a member of the vesicle associated membrane protein family (VAMP). Synaptobrevin is a vesicle-SNARE protein, a family of proteins that directly mediate the fusion of synaptic vesicles with the cell membrane (Edelmann, 1995). Thus, synaptophysin is a presynaptic protein directly associated with vesicle release machinery.

Increased levels of synaptophysin have been correlated with learning or increased task performance in a number of paradigms. Environmental enrichment and

improvements in spatial memory acquisition are correlated with increased cortical and hippocampal synaptophysin levels (Frick, 2002). In the fear system, auditory fear conditioning is known to increase levels of synaptophysin in the LA (Nithianantharajah, 2008). Moreover, these researchers used an increase in synaptophysin levels as evidence of synaptic plasticity.

This current study

The results of this dissertation have shown that the LA is significantly regulating training induced EGR-1 upregulation in the auditory thalamus. This EGR-1 upregulation is, in turn, required for fear memory consolidation. EGR-1 is an IEG that acts as a transcription factor, meaning that its upregulation leads to the subsequent increase of genes that contain EGR-1 binding sites. Such genes include the presynaptic proteins synapsin I, II (Petersohn, 1995; Theil, 1994). In addition, EGR-1 upregulation is correlated with an increase in the presynaptic protein synaptophysin (Matsubara, 2001; Rossino, 1995). Thus, it seems plausible that EGR-1 upregulation in the auditory thalamus could subserve presynaptic alterations in the LA. Indeed, previous research has shown that fear conditioning results in LA localized increases in the presynaptic protein synaptophysin (Nithianantharajah, 2008).

The aim of this current study was to, firstly, determine whether auditory fear conditioning would increase protein levels of the synapsins and synaptophysin in the LA. Thus, in part, the aim was to replicate the findings of Nithianantharajah and colleagues (2008). As described above these proteins play a key role in regulating synaptic vesicle release at the presynaptic membrane. Thus, it is hypothesized that fear conditioning should increase the levels of these presynaptic constitutes.

A subsequent aim of this experiment was to determine whether conditioned induced increases in these synaptic proteins were downstream of training induced EGR-1 upregulation in the auditory thalamus. Animals received intra-thalamic microinfusions of EGR-1 ODN or scrambled control prior to training. If training induced EGR-1 upregulation is, in fact, controlling the expression of these proteins within the LA then animals that received intra-thalamic EGR-1 ODN microinfusions should show significant lower levels of these proteins 24-hours later compared to animals that received microinfusions of scrambled control.

Method

Experiment 1: Time course analysis of presynaptic proteins synapsin and synaptophysin upregulation in the LA

Subjects

Twenty two adult (300-350g) male Sprague Dawley rats (Hilltop Laboratories, Philadelphia, PA) served as subjects. They were housed individually in plexiglass cages in a vivarium maintained on a 12 h light/dark cycle. All procedures were conducted during the light phase of the cycle. Food was available *ad libitum* throughout the experiment.

Behavioural procedures

The day before training animals' were habituated to handling. On training day, animals were randomly assigned to one of three groups. A naïve group that simply remained in their home cages, a 6-hr time point group, and a 24-hr time point group. The animals in the latter two groups were placed in the conditioning chamber and allowed 150 sec to acclimate. They were then presented with 10 tone-shock pairings at an average ISI of 120 seconds. The tone (20 s, 5kHz, 75 dB) was presented for 20 sec and coterminated with a 1 sec 1 mA footshock.

After training the animals were sacrificed at the appropriate time points that corresponded with the assigned group, 6 hours and 24 hours post training. Animals' received an overdose of chlorohydrate (250 mg/kg, I.P. administration). The brains were rapidly frozen at -80°C until processing. See Figure 32 for a schematic of the behavioural protocol used in this experiment.

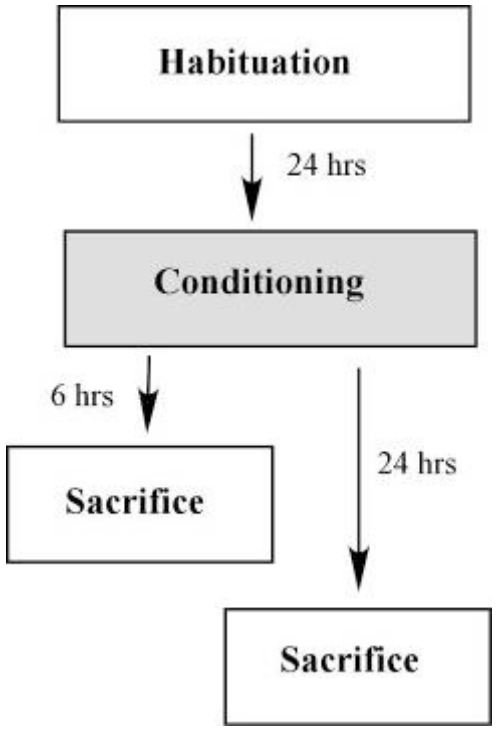

Figure 32. Schematic of the behavioural procedure used to examine the upregulation of the presynaptic proteins synapsin I, synapsin II and synaptophysin. Animals were habituated to handling 24 hrs prior to conditioning. Animals were then conditioned and sacrificed 6 or 24 hrs post-training. LA sections were then analysed for the immunoreactivity of the above mentioned presynaptic proteins.

Immunoblotting procedure

Punches containing the LA were obtained with a 1 mm punch tool (Fine science Tools, Foster City, CA) from 400-µm-thick frozen sections taken on a sliding microtome. Punches were manually dounced in 100 µl of ice-cold hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, 1mM EDTA, 2.5 mM sodium pyrophosphate, 1mM phenylmethylsulfonyl fluoride, 1mM β-glycerophosphate, 1% Igepal CA-630, 1%

protease inhibitor cocktail (Sigma) and 1 mM sodium orthovanadate). Sample buffer was immediately added to the homogenates, and the samples boiled for 6 min. Homogenates were electrophoresed on 10% Tris-HCl gels and blotted to Immobilon-P (Millipore, Bedgord, MA). Western blots were blocked in TTBS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) with 5% dry milk and then incubated with either anti-synapsin antibody (1:1,000: Cell Signalling), or anti-synaptophysin antibody (1:5,000: Dako Cytomation). Blots were then incubated in an anti-rabbit secondary antibody conjugated to horseradish peroxidase (Cell Signaling) and developed using West Dura chemiluminescent substrate (Pierce Laboratories, Rockford, IL). Densitometry was conducted using Image J software. To control for inconsistencies in loading, optical densities were normalized to (glyceraldehyde-3-phosphate dehydrogenase) GAPDH protein (1:5,000; Abcam).

Data Analysis

Data were normalized to the average value of vehicle-controls and results were analysed using ANOVA.

Experiment 2: Thalamic EGR-1 regulation of presynaptic proteins: Synaptophysin, Synapsin I and Synapsin II in the LA

Subjects

Eleven adult (300-350g) male Sprague Dawley rats (Hilltop Laboratories, Philadelphia, PA) served as subjects. They were housed individually in Plexiglas cages in a vivarium maintained on a 12 h light/dark cycle. All procedures were conducted during the light phase of the cycle. Food was available *ad libitum* throughout the experiment.

Surgery

Surgery protocols were identical to those described in previous chapters. The cannulae for this experiment, however, were 26 gauge (Plastics One, Roanoke, VA) and were aimed at the auditory thalamus (AP: 5.5, ML: +2.8, DV: -6.6).

Oligodeoxynucleotide design and preparation.

The same EGR-1 ODN that was used for the research described in Chapter 5 was also used in this experiment.

Behavioural procedures

The day before training the animals were habituated to handling and cannula insertion. On training day, animals were randomly divided into Antisense-ODN (n=6) and Scrambled-control (n=5) groups. Rats were then handheld and the dummy cannulas removed, infusion cannulae which protruded 2 mm below the tip of the guides were inserted. The appropriate drug was then bilaterally infused. After infusion was complete the cannulae were retained in the guide for a further 2 min to allow drug diffusion from the tip.

Ninety minutes following drug infusion the animals' were placed in the conditioning chambers. The conditioning procedures were identical to those utilized in the experiment described above. Briefly, animals were given 150 sec to acclimate and then presented with 10 tone (20 s, 5kHz, 75 dB) shock (1 sec, 1 mA) pairings.

Animals were sacrificed 24 hours after training by an overdose of chlorohydrate (250 mg/kg, I.P. administration). The brains were rapidly frozen at -80°C until processing. See Figure 33 for a schematic of the behavioural procedures used in this experiment.

Brains and subsequent data analysis were processed in exactly the same manner as described above.

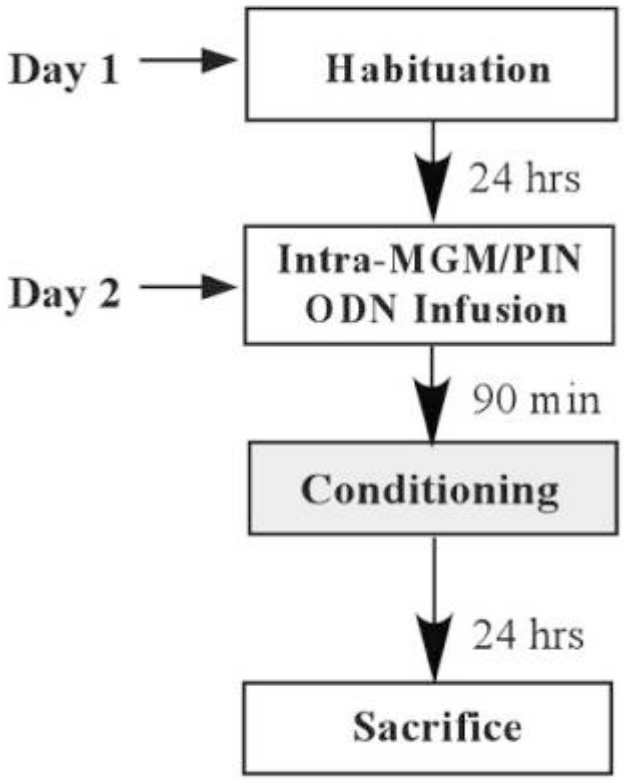

Figure 33. Schematic of the behavioural procedures used to examine the upregulation of synapsin I, synapsin II, and synaptophysin in the LA as a function of intra-thalami knock-down of EGR-1. Animals were habituated to handling the day before training. 90 min prior to training animals received intra-thalamic microinfusions of EGR-1-ODN. Animals were then conditioned. 24-hrs post conditioning the animals were sacrificed and LA tissue analyzed for the immunoreactivity of the presynaptic proteins of interest.

Results

Experiment 1: Protein levels of synaptophysin, synapsin I and synapsin II are significantly increased in the LA 24 hours post conditioning

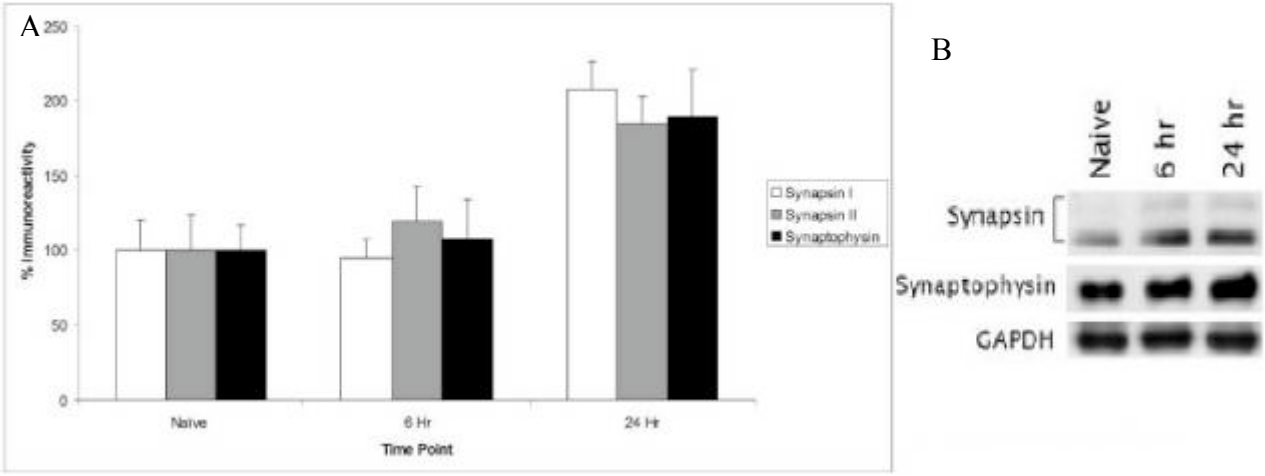

Figure 34. **A.** Percent immunoreactivity (± SEM) of synapsin I and II, and synaptophysin in LA homogenates in animals who received ten tone shock pairings and then sacrificed either 6 hr or 24 hr post training or functioned as naïve controls. * $p<0.05$. **B.** Representative immunoblots showing increased immunoreactivity of presynaptic proteins over the time-course.

Figure 34 shows the average % immunoreactivity of protein in samples obtained from the LA in animals sacrificed at 6- and 24-hr time points relative to a naïve control. The results show an increase in the level of synaptophysin, synapsin I and synapsin II protein immunoreactivity in the 24-hr group compared to both the Naïve control and 6-hr point. A 3(Time point: Naïve, 6-hr, and 24-hr) x 3 (Protein: Synaptophysin, Synapsin I and Synapsin II) repeated measures ANOVA (with protein as the repeated measure) yielded a significant effect of time point ($F(2,13)=8.21$,$p<0.005$), while a significant effect of Protein ($F(2,26)= 0.02$,$p>0.9$) or a Protein X Time point interaction ($F(4,26)=0.65$,$p>0.6$) was not revealed. Post-hoc analysis revealed that the 24-hr group differed significantly from both the Naïve and 6-Hr animals ($p<0.004$, Dunnett's t) Thus, showing that the proteins were equivalently regulated at each time point with a significant increase in protein expression at the 24-hr point.

Experiment 2: Thalamic EGR-1 upregulation is required for training induced increases in the presynaptic proteins synaptophysin, synapsin I, and synapsin II.

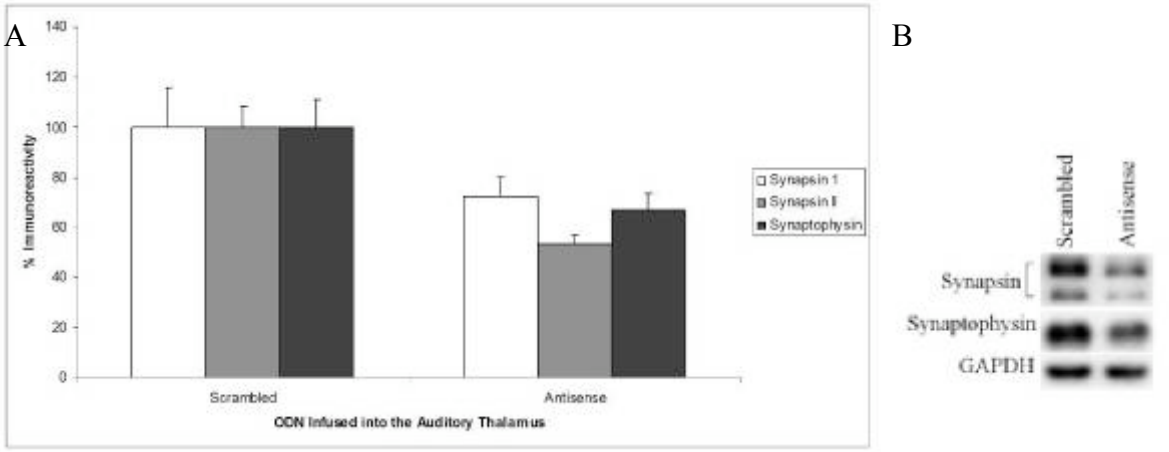

Figure 35. **A.** Percent immunoreactivity in LA homogenates of the presynaptic proteins synapsin I, synapsin II and synaptophysin as a function of intra-thalamic knock down of EGR-1 expression. Animals received intra-thalamic microinfusions of EGR-1 antisense ODN or scrambled control prior to training with ten tone shock pairings. Animals were then sacrificed 24-hrs post training and the immunoreactivity of selected presynaptic proteins determined. *$p<0.05$ **B.** Representative immunoblots showing reduced density of presynaptic proteins in the antisense condition compared to scrambled controls.

Figure 35 shows the percent immunoreactivity of the presynaptic proteins synapsin I, synapsin II and synaptophysin 24-hr post-conditioning as a function of intra auditory thalamic microinfusions of EGR-1 ODN-antisense or scrambled control. The graph shows that the levels of all three of the proteins were reduced in the Antisense group when compared to the Scrambled controls. A 2(Drug: Antisense vs. Scrambled) X 3(Protein: Synaptophysin, Synapsin I, and Synapsin II) repeated-measures ANOVA with protein as the repeated factor did, indeed, reveal a significant effect of Drug ($F(1,9)=15.99, p<0.004$). While a main effect of Protein ($F(2,18)=0.25, p>0.5$) or a significant Drug X Protein interaction ($F(2.18)=0.25, p>0.05$) was not observed. These results indicate that all three proteins were equivalently reduced in the EGR-1 ODN group compared to the Scrambled Controls.

Discussion

The aim of this experiment was to determine whether the activity of intracellular pathways in the auditory thalamus serves to support presynaptic alterations locally in the LA. Firstly, this series of experiments examined whether fear conditioning regulates the expression of the presynaptic proteins synaptophysin, synapsin I, and synapsin II. The results revealed that these three proteins were all equivalently and significantly regulated by training as indicated by a significant increase in their LA levels 24-hours post-training. In the next phase of the experiment an examination of whether training-induced EGR-1 upregulation in the auditory thalamus regulates this increase in presynaptic proteins within the LA was conducted. The selective EGR-1 antisense ODN was microinfused into the auditory thalamus prior to conditioning followed by an examination of the affect this had on training-induced regulation of presynaptic proteins 24-hours post training. Results indicated a significant reduction in the expression of synaptophysin, synapsin I and synapsin II in the animals who received intra thalamic ODN-EGR-1 microinfusions compared to those who received scrambled control. Importantly, our lab has shown that intra-thalamic microinfusions of EGR-1ODN irrespectively of training does not reduced LA based levels of synaptophysin, synapsin I or II, indicating that only training induced upregulation of these proteins was reduced via this manipulation. Overall, this experiment has shown that EGR-1 upregulation in the auditory thalamus is upstream of training induced increases in the presynaptic proteins synaptophysin, synapsin I and synapsin II, thus, showing that EGR-1 serves to support synaptic efficacy within the LA.

Previous research has shown that inhibition of ERK activity in the auditory thalamus impairs fear memory consolidation (Apergis-Schoute, 2005). Moreover, inhibition of ERK in the PP/PIN/MGM prevents the induction of LTP-induced plasticity in the LA, indicating that activation of such intracellular pathways in the auditory thalamus serves to support the formation of an LA-based association (Schafe, 2005). This research has shown that this does indeed appear to be the case as evidenced by an increase in presynaptic proteins in the LA downstream of EGR-1 signalling in the auditory thalamus. Additional research could be conducted in order to specifically examine whether this alteration in presynaptic proteins corresponds with an increase in electrophysiological responding at thalamo-LA synapses. More specifically, consistent with the methods used by Schafe and colleagues (2005), an EGR-1-ODN could be microinfused into the auditory thalamus prior to training, electrophysiological changes that occur in the LA as a function of training could be monitored. If the presynaptic proteins that lie downstream of EGR-1 upregulation in the auditory thalamus are indeed contributing to training-induced plasticity in the LA then such a manipulation should reduced training induced CS evoked potentials in the LA. Furthermore, in order to understand the protein components pertinent for the formation of an auditory fear memory association analysis of whether or not upregulation of these presynaptic proteins is required for conditioning via knock down of the expression of the corresponding genes is required.

When considering the results of this current study in light of the previous research findings of this dissertation a clear cell signalling pathway at the LA-Thalamo synapses emerges. The results of this dissertation have shown that activation of NR2B-NMDA receptors in the LA leads to the synthesis of NO, which acts as a retrograde signalling molecule to modulate intracellular pathways in the presynaptic cell. More

specifically, NO synthesis ultimately leads to the upregulation of EGR-1 in the auditory thalamus. The training induced upregulation of this IEG is, in turn, necessary for auditory fear memory consolidation. Further, unpublished observations from our lab have shown that 1) PKG upregulation is, at least in part, regulating auditory thalamic EGR-1 upregulation and that 2) ERK induced modulation of EGR-1 activity in the auditory thalamus has been observed. The results of this current research has shown that EGR-1 upregulation, in turn, results in an increase in presynaptic proteins locally within the LA, this potentiation of presynaptic machinery is thought to, in turn potentiate synaptic connectivity at Thalamo-LA synapses (see Figure 36).

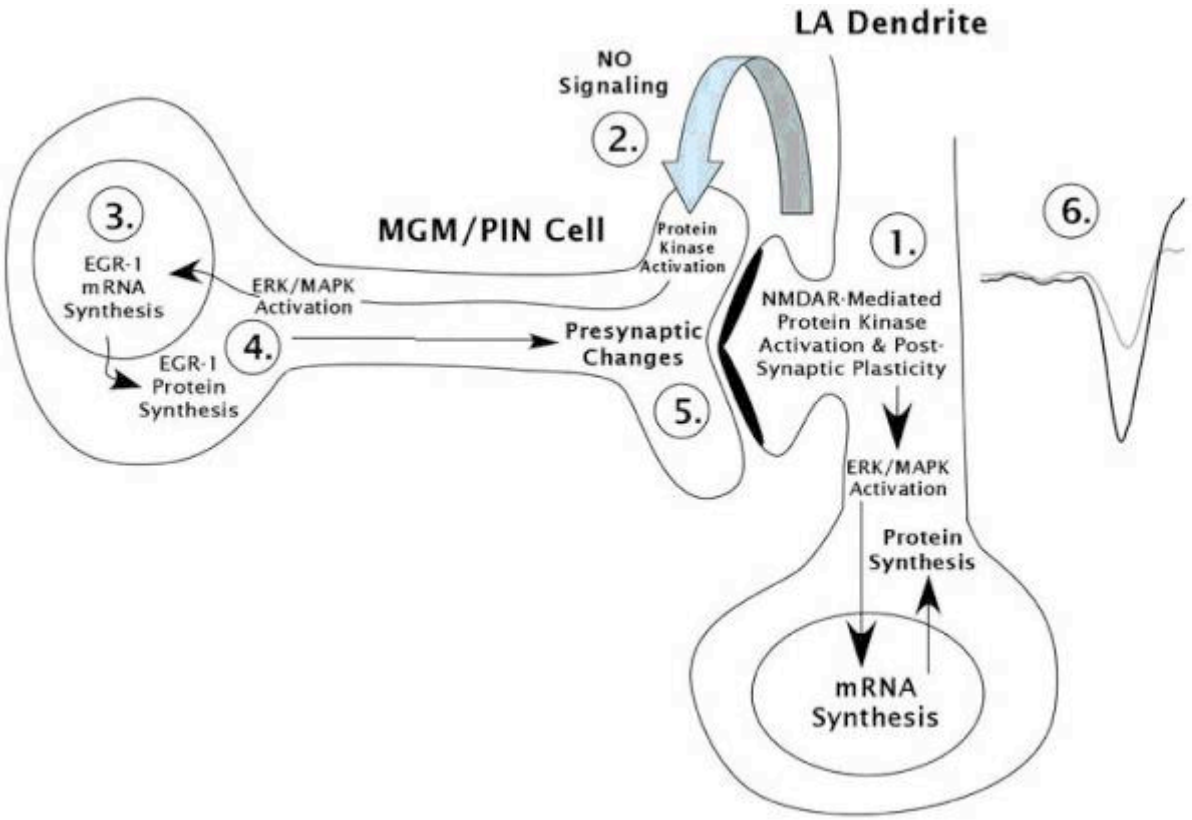

Figure 36. Schematic representation of a thalamo-LA synapse. The schematic depicts the intracellular processes that are thought to underlie synaptic potentiation/ fear memory consolidation at these synapses. 1) Activation of NMDA receptors on postsynaptic LA spines results in calcium influx through the receptors that leads to activation of intra cellular pathways within the postsynaptic cell. This includes activation of nNOS, which results in the synthesis of 2) NO- a retrograde signalling molecule that activates intracellular pathways in the presynaptic MGM/PIN cell. More specifically, activation, at least in part, of PKG leads to ERK/MAPK activation, which is upstream of 3) EGR-1 upregulation. Training induced EGR-1 upregulation is, in turn, necessary for fear memory consolidation. It appears that it functions, in part, to increases the density of presynaptic proteins at thalamo cell terminals located within the LA. This increase presynaptic machinery theoretically leads to 6) an increase in synaptic connectivity at the LA-thalamo synapse.

Chapter 7

General Discussion and Conclusions

Pavlovian fear conditioning is a behavioural paradigm used by researchers as a tool to understand the neuroanatomical underpinnings of both fear related psychopathology and associative memory formation. It is generally accepted that the LA is a key neuroanatomical locus of the memory representation. In the research conducted for this thesis auditory fear memory conditioning was the conditioning paradigm of choice. During the procedure, an animal is presented with an emotionally neutral CS: a tone, in the presence of an innately fear US: a footshock. The LA receives incoming CS and US associated neurotransmission from thalamic nuclei and cortical processing areas. This convergence of information is believed to potentiate CS-associated synapses. As a result, subsequent presentation of the CS, in the absence of the US, results in a fear response. It can be said that the CS has now acquired the emotional value of the US. It can now excite cells of the LA to a level that ultimately results in a fear response via activation of the cells in the CE.

Over the last ten years or so there have been tremendous advances in the understanding of cellular and molecular events that underlie the potentiation of synaptic connections pertinent for fear memory formation. Briefly, the general doctoring follows, that an increase in intracellular calcium concentration sets in motion cell signalling cascades that begin with activation of second messengers. These, in turn, activate proteins kinases that further propagate the signal into the cell. As well as being responsible for STM, kinase signalling cascades enter into the nucleus of the neuron and activate transcription factors. These, gnomically modify the cell by initiating, or increasing, the transcription of various genes. The first genes upregulated are the IEG's.

These genes are translated into proteins that either, as new protein components, change the protein constitutes within the cell, or operate as transcription factors that return to the nucleus of the cell to upregulate late response genes. Overall, this change in genomic activity and subsequent protein synthesis is thought to alter the structure and function of the cell. Specifically, this is thought to occur at synapses that form the fear memory engram. For example, memory formation is associated with growth at the postsynaptic density. An increase in postsynaptic proteins such as excitatory receptors, in turn, leads to an increase in synaptic efficacy. However, research concerning associative memory formation has predominantly centred on processes occurring in the postsynaptic cell. While the role of the presynaptic cell has not been completely disregarded- it is generally believed that an increase in postsynaptic efficacy is associated with an increase in presynaptic functioning. However, a detailed delineation of this process where upstream intracellular signalling mechanisms' are investigated, especially in a behavioural model, is lacking.

The hypotheses of this thesis were as follows: firstly, during auditory fear conditioning the LA is significantly modulating presynaptic activity, or more specifically intracellular pathways within the auditory thalamus. Given that inhibition of NO signalling in the LA impairs fear memory consolidation, it was secondly, hypothesised that the LA is modulating presynaptic activity via this retrograde second messenger signalling molecule. Further, previous research has revealed that plasticity within the auditory thalamus is required for the formation of conditioned-induced plasticity within the LA (Schafe, 2005). This suggests that if the LA is modulating intracellular pathways within the auditory the third hypothesis follows, that it does so in order potentiate synaptic communication back at LA synapses.

Within this dissertation Chapters 2-6 describe the series of experiments that were conducted. Each of these chapters contained a comprehensive discussion relating to each experiment. The following concluding chapter begins with a brief review of the research findings followed by a discussion with more breadth than the sub discussions in the previous chapters. It includes research limitations, how the results of this research relate to the current understanding of memory formation and future perspectives.

Summary of findings

The current research began by examining whether auditory fear conditioning increased EGR-1 protein levels in the PP, PIN, and MGM nuclei of the thalamus (Chapter 2). The results of this experiment revealed a significantly greater degree of EGR-1 upregulation in Paired animals when compared to a Tone Alone or Naïve control. On the other hand, EGR-1 upregulation in the Immediate Shock or Unpaired group, while being less than animals exposed to a paired protocol, did not differ significantly from this conditioned group. However, it is likely that this result was confounded by the analysis of EGR-1 upregulation across the auditory thalamus as a whole, irrespective of the specific neuroanatomical network that underpins an auditory fear memory association.

The aim of the second series of experiments (Chapter 3) was to determine whether pharmacological manipulation within the LA would interfere with training induced EGR-1 upregulation in the auditory thalamus. In brief, the results revealed that the LA plays a significant role in EGR-1 upregulation in the auditory thalamus during the acquisition phase of fear memory formation via the retrograde signalling molecule NO. Given that training induced EGR-1 upregulation in the auditory thalamus is

modulated by LA activation the results of Chapter 2 were readdressed. However, the analysis was restricted to auditory thalamic cells that project to the LA.

Next the retrograde signalling molecule FG was used to localize cells in the auditory thalamus that project to the LA. Quantification of EGR-1 reactivity within this subsection of neurons, which most importantly constitute a key component of the auditory fear memory engram, produced a significant difference in EGR-1 upregulation in Paired group when compared to an Immediate-shock and Naïve control (Chapter 4).

Finally, it was revealed that auditory thalamic EGR-1 upregulation is required for consolidation, but not STM formation, of an auditory fear memory association (Chapter 5). Further it appears that is may necessitate fear memory formation, at least in part, by increasing levels of the presynaptic proteins synapsin I, synapsin II, and synaptophysin within thalamic cell terminals located in the LA (Chapter 6).

Overall, this PhD research has shown that NO synthesis in the LA during fear memory formation leads to an upregulation of the IEG EGR-1 in auditory thalamic cell bodies, which in turn acts to alter presynaptic proteins levels back within the LA. These presynaptic proteins play a role in vesicle release so it could be assumed that NO signalling in the LA serves to potentiate synaptic transmission at thalamic cell terminals.

Research Limitations

As with any dissertation research time was a huge deficit when considering the research undertaken in this thesis. As a result some of the group sizes were substantially smaller than would have been desired. While my analyses did consistently produce significant results there were some experiments where an increase in sample size may have produced much cleaner results. More specifically, the results of Chapter 2, where

training induced EGR-1 upregulation in a Paired group failed to reach significance when compared to an immediate shock and un-paired control, may have reached significance if the sample size of these groups was increased. The reason behind this assumption is the fact that the Paired group did indeed have a greater EGR-1 count, just not at a significant level. Further, post-hoc analysis revealed that Immediate Shock and Unpaired controls did not differ significantly from the Tone alone and Naïve groups. While a number of theoretical explanations' for this result have been mentioned in detail within Chapter 2 it is possible that some methodological differences may have moved the results in a more desirable direction. For example, as briefly discussed in Chapter 2, Malkani and Rosen (2000) examined training induced EGR-1 upregulation in the LA. In their series of experiments, however, the animals underwent five days of habituation to handling. It has been suggested that such habituation could desensitizes gene upregulation to stimuli that at the beginning of the experiment were novel (Tischmeyer, 1999). That is, the act of taking the animals from their housing environment to the testing environment and handling by the experimenter could drive gene upregulation. In the experiments conducted for this dissertation the animals received one day of habituation. It may have been advantageous for the results of this study to have habituated for more days, not only in the experiment conducted in Chapter 2 but all the subsequent experiments. However, it should be emphasised that this discussion should not detract from the fact that the results indicated significant differences in the experiments especially when gene regulation across the auditory thalamus as a whole was conducted; they may have been consistently significant across all nuclei of the auditory thalamus, however, if additional days of habituation had occurred.

A further limitation to be considered is that within most of the experiments fear memory formation was not assessed. Throughout the experiments 1-10 conditioning trails were used. While there are countless numbers of research findings illustrating that as little as one conditioning trail is substantial enough to produce a robust fear memory association, it still would have been advantageous to include control groups to assess whether conditioning occurred or not during the different experiments. More specifically, in Chapter 3 an examination of thalamic EGR-1 upregulation as a function of LA based pharmacological manipulations' was conducted. To this end, pharmacological manipulations previously shown to impair various processing levels of fear memory formation were employed. More specifically, the first experiment examined intra LA $GABA_A$ receptor antagonism, which prevents cellular excitation in general and thus fear related neurotransmission. Consequently simple processing of US-induced fear is impaired. NO antagonism at two levels was examined, each of which is known to impair LTM formation. Antagonism of ERK/MAPK was employed, which is specifically known to impair LTM but not STM. Finally, NR2B-NMDA receptor activation was impaired, which is known to impair fear memory acquisition but not fear related neurotransmission. The point is, in all of these experiments it would have been interesting to include a control group that received the same pharmacological manipulations but instead of being sacrificed 2-hour post conditioning, as occurred with the experimental groups in order to analyses EGR-1 upregulation, the animals STM and LTM could have been assessed over the 24 hours post training. This additional manipulation would have been an eloquent control, assuring that in my hands these pharmacological manipulations produced the anticipated affect. Nonetheless, given the reported thalamic EGR-1 upregulation induced after each of the intra-LA manipulations it seems probable that the drugs would have produced the desired affects.

Contribution of this research to the current literature and future perspectives

This dissertation research, as far as I am aware, is the first of its kind to demonstrate that NO serves as a retrograde signalling molecule in a behavioural memory model. Here the results have shown that NO does indeed act as a retrograde signalling molecule bridging the gap between cellular excitation in the LA and activation of intracellular pathways in the auditory thalamus. The results have indicated that this activity leads to potentiation of presynaptic proteins back in the LA. Presumably this potentiation is occurring at synapses where NO was originally synthesised.

Nitric oxide is a diverse regulator of gene expression. Future research is required to examine the breadth of its signalling contribution to gene upregulation in fear memory formation. For example, the NO pathway has been implicated in amygdala BDNF upregulation in the fear related shuttle avoidance task (Chein, 2008). Whether NO modulates BDNF expression during fear conditioning and whether it is pertinent for fear memory consolidation needs to be investigated. Further, the role NO plays in other memory paradigms besides Pavlovian fear conditioning needs to be investigated. Perhaps its downstream targets that were observed in this research are consistent across all brain structures and memory paradigms, or perhaps there are some important differences.

The results of this research have also revealed some of the dynamics of EGR-1 signalling. The results indicated that EGR-1 upregulation serves, at least, in part, to alter cellular structure within cell terminals. EGR-1 upregulation occurs in a number of *in vivo* and *in vitro* memory paradigms. It is probable that EGR-1 activity in these paradigms also serves to support plasticity at neuronal terminals. Further, given its role

as a transcription factor, it is likely that EGR-1 upregulation is upstream of a number of other late response genes. The protein components of such genes could modify other structural and functional aspects within a neuron. Its ability to modulate the activity of such genes could also be pivotal memory formation.

In general, research concerning the structural or functional consequence of IEG upregulation is presently lacking. More specifically, current research concerning gene regulation, especially IEG regulation, has focused on how various stimuli contingencies within behavioural paradigms, or electrophysiological manipulations in *in vitro* memory models, alter IEG expression. It is generally agreed that gene expression within the nervous system serves to change the functioning of the neuron involved. In order to fully understand the functional role of various genes it is necessary to go beyond correlation of gene expression with experimental manipulations and examine the eventual structural, physiological, and behavioural consequence of modified genomic expression.

Presynaptic potentiation at other synapses within the fear neurocircuitry

The research conducted for this dissertation examined presynaptic aspects of fear memory formation at thalamo-LA synapses. It would be interesting to examine whether retrograde signalling at other synapses within the fear circuitry is also important for fear memory formation. For example, there are cortical projection cells within auditory thalamic nuclei. Perhaps NO signalling from cortical auditory association areas is contributing to EGR-1 upregulation in the auditory thalamus. Or upregulation of numerous other IEG's for that matter. Further, there are other means of cellular retrograde signalling, such as transynaptic protein-protein interactions or signalling via the natriuretic peptides. (Decker, 2008; Futai, 2007). For example,

neuroligins are transmembrane proteins expressed on the postsynaptic cell that bind to ß-neurexins, which are presynaptic transmembrane proteins. Recent research has shown that silencing of endogenous neuroligin-1 within the LA reduces NMDA channel conductance and prevents LTP without interfering with normal synaptic connectivity (Kim, 2008). Such a finding illustrates how connectivity between the pre- and postsynaptic-cell activity is necessary for an increase in synaptic efficacy. The specific dynamics of this interaction is yet to be determined. Specifically, whether there is a change in the connectivity of thalamo-LA synapses as a result of memory formation.

The natriuretic peptides are also known to act as retrograde signalling molecules and are able to activate membrane bound particulate GC. As with NO they have been reported upstream of EGR-1 upregulation (Thiriet, 1997). Within the fear circuitry messenger mRNA for the atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP) have been localized in the CE (Langub, 1995; Quirion, 1986), indicating that these retrograde signalling molecules could coordinate, or at least modulate, plasticity associated activity between LA-CA synapses. Indeed, EGR-1 upregulation in the LA is necessary for contextual fear memory formation (Malkani, 2004). Perhaps retrograde signalling from the CE via the natriuretic peptides may be contributing to this LA based EGR-1 upregulation.

Clinical relevance of this research

The amygdala plays a role in other psychological processes such as the processing of reward and, in line with this, drug addiction (LeDoux, 2007). Less is known about the amygdala based neuronal circuitry that underlies these processes. It is possible that a LA based modulation of presynaptic functioning plays a role. Moreover, the findings of this research may help to delineate the molecular underpinning of

psychological disorders associated with exaggerated fear and anxiety. Research such as that conducted for this dissertation while focussing on “normal” memory processing nonetheless provides a wealth of knowledge to the literature concerning such fear related psychopathology. As researchers strive to understand fear memory processing they highlight many neuronal processes that could in be exaggerated or inadequate in a patient suffering from fear related psychopathology. Overall, the current study provides new insights into the molecular bases of fear memory formation and illustrates the significant role played by NO in mediating presynaptic activity during this process.

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